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The effect of cations on trypsin kinetics using [DL-]BAPNA as the substrate

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THE EFFECT OF CATIONS ON TRYPSIN KINETICS USING BAPNA AS THE SUBSTRATE

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ABSTRACT

THE EFFECT OF CATIONS ON TRYPSIN KINETICS USING DL-BAPNA AS THE SUBSTRATE

by Sathyappriya Pichaikannu

The effect of the cations Ca (II), Mn (II), K (I), Ce (III), Cr (III), Mg (II) on the catalytic activity and secondary structure of trypsin was studied using DL-BAPNA as the substrate. Values of K_M and k_{cat} for the reaction of the cations with trypsin were determined. Among the six metal ions only Ca (II) and Ce (III) showed some effect on the activity of trypsin. Though there was a 17 % increase in activity of trypsin for 0.5 M Ca (II), the results were not statistically significant due to overlapping of the error bars. There was a 66% enhancement in the activity of trypsin at 0.5 mM Ce (III) concentration. At higher concentrations, cerium showed no activity for trypsin. The values of K_M and k_{cat} for 0.5 mM Ce (III) were determined to be 1.8 mM/Liter and 0.058 sec^{-1}, respectively. The far ultraviolet CD spectra of trypsin with 0.005-0.5 mM Ce (III) and Ca (II) were the same as that of the control. Thus, the cations, Ca (II) and Ce (III), do not show any effect on the secondary structure of trypsin.

Acknowledgements

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CHAPTER 1.0

INTRODUCTION

1.1 General Background

Proteins are made up of amino acids. Each amino acid contains at least one carboxyl group and one α-amino group, but they differ from each other in the structure of their side chains. Proteins have diverse biological functions. Enzymes are proteins of high molecular weight that act as catalysts. They accomplish their functions through various mechanisms that depend on the arrangement of functional groups in the enzyme's active site where catalysis occurs [1, 2]. Figure 1 shows the active site of an enzyme where the substrate gets cleaved to the product.

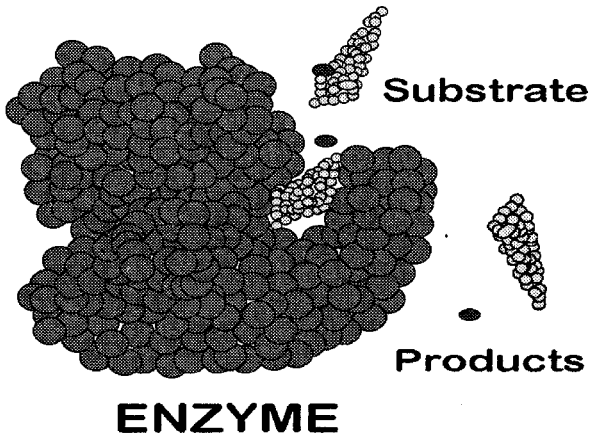


Figure 1. Active site for an enzyme where catalysis occurs [3].

Kinetic studies of enzymes have been conducted for decades and are a powerful tool for determining enzyme activity. Kinetic data along with information about an enzyme's structure could provide some important clues to the enzyme's biological function and may suggest ways to modify the enzyme for therapeutic purposes [1]. Kinetics of many enzyme-catalyzed reactions follow the Michaelis-Menten model:

$$V = \left(\frac{V_{\max}[S]}{K_M + [S]} \right) \qquad \text{Equation 1}$$

where V is the rate of the reaction, V_{max} the maximum rate, [S] the substrate concentration and K_M the Michaelis-Menten constant. Figure 2 shows the plot of substrate concentration versus reaction rate. The substrate concentration corresponding to half the maximum rate is the Michaelis - Menten constant, K_M.

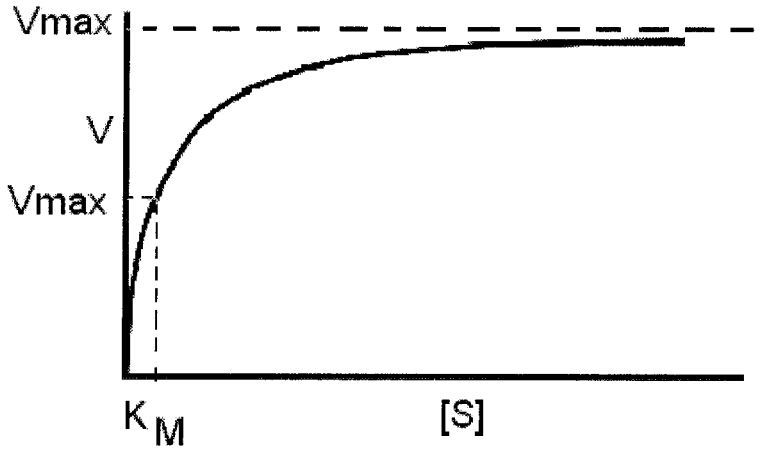


Figure 2. Plot of substrate concentration versus reaction rate [4].

Use of synthetic compounds as substrates for enzymes has considerably simplified the procedure for the determination of enzymatic activity. Chromogenic substrates for serine proteases include the p-nitroanilides and p-nitro phenyl esters of free or N-acylated amino acids and peptides. These substrates produce a chromogen on enzymatic hydrolysis, which can be quantitatively estimated spectrophotometrically [5]. The UV and visible spectra of a biomolecule produced by a spectrophotometer reveal much about the molecular structure of the biomolecule and serve as a valuable tool for the characterization and analysis of biologically significant molecules and dynamic processes occurring between them [6].

In order to predict the characteristics of the secondary structure of proteins, an ultraviolet circular dichorism (CD) spectrum can be used. The CD spectrum of a biomolecule is used to estimate the fraction of a molecule that is in the alpha-helix conformation, the beta-sheet or beta turn conformation or some other random conformation. From this information one can predict the possible secondary conformations of the protein. It can also be used to predict the changes in conformation when the protein is subjected to changes in temperature or mixed with different concentrations of denaturing agents and salts [7]. Different types of protein secondary structures (helices, sheets, turns and coils) give rise to different CD spectra as shown in Figure 3. Therefore, it is also possible to extract the secondary structure information for an unknown protein from its CD spectrum [7].

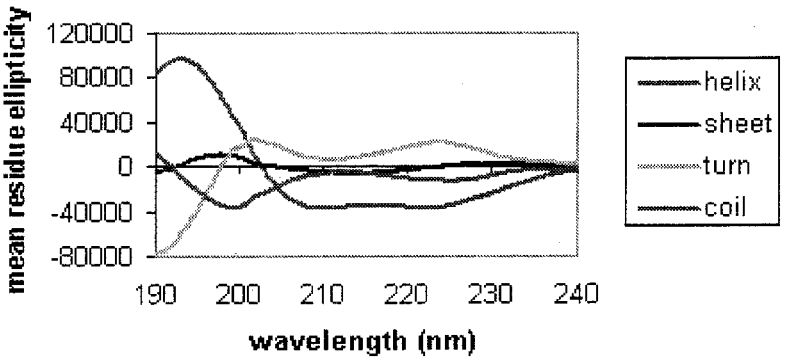


Figure 3. CD spectra of different types of protein secondary structure [7].

1.2 Trypsin

Trypsin is one of the three main serine proteases, which helps in breaking down dietary proteins into their corresponding peptides and amino acids. Trypsin's substrate specificity is based on positively charged lysine and arginine residues and shows maximal enzymatic activity in the small intestine where it is present under slightly alkaline conditions (approximately pH=8) [8].

The study of trypsin kinetics is becoming increasingly important since trypsin is used widely in many applications, such as: a) in cell culture for research and production of recombinant proteins for clinical uses; b) in the wound care markets as an oral treatment for inflammatory edema, hematoma, and pain associated with a wide variety of internal and external wounds; c) in industrial applications [9]. Its major application is in the field of proteomics. Trypsin is targeted for the digestion of proteins before mass spectrometric analysis. Since trypsin has a high specific activity, the digestion time for proteins is greatly reduced, resulting in a smaller amount of auto digest fragments of trypsin. A reduced digestion time and a smaller amount of auto digest fragments of trypsin leads to a higher accuracy in mass spectrometry [10].

Trypsin is a critical intermediary in the manufacture of insulin, where it is used to help cleave the zymogen into its active form [11]. Figure 4 shows the amino acids histidine, serine and aspartate present in the catalytic region of trypsin called the catalytic triad.

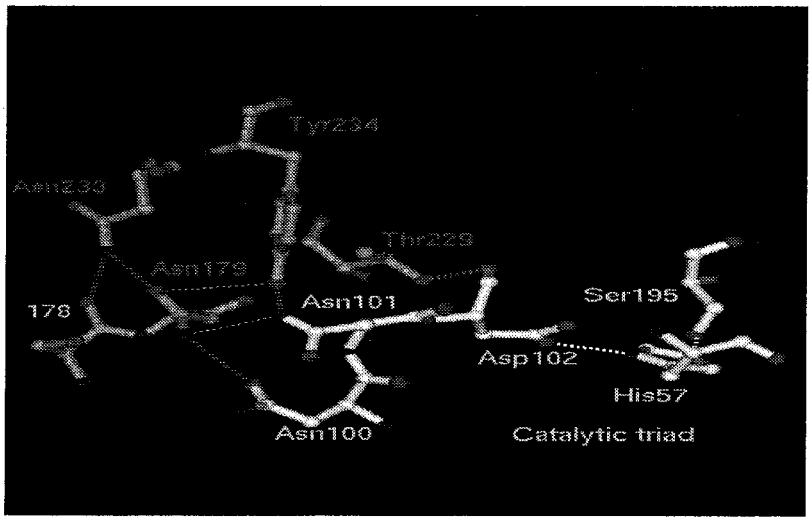
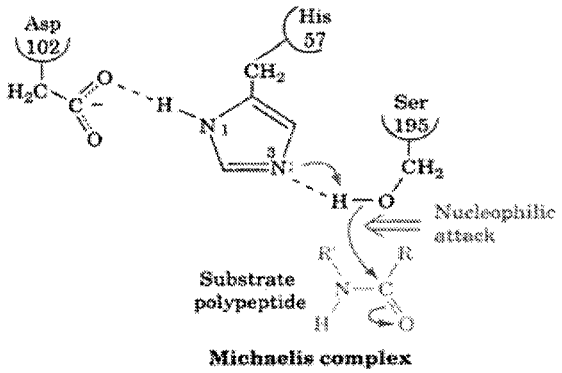


Figure 4. Amino acids present in the catalytic region of bovine pancreatic trypsin [11].

The catalytic mechanism of trypsin involves the following reaction: (1) the nucleophilic attack of the serine on the carbonyl carbon of the scissile (to be cleaved) peptide bond to form the tetrahedral intermediate [1]; (2) the decomposition of the tetrahedral intermediate to the acyl-enzyme intermediate, followed by loss of the amine product and its replacement by a water molecule; (3) the reversal of step 2 to form a second tetrahedral intermediate, and (4) the reversal of step 1 to yield the carboxyl product and the active enzyme [1]. Figure 5 shows the steps involved in the catalytic mechanism of trypsin.



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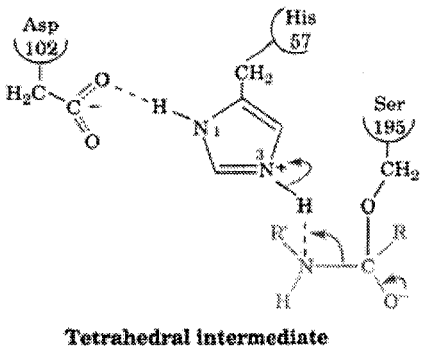

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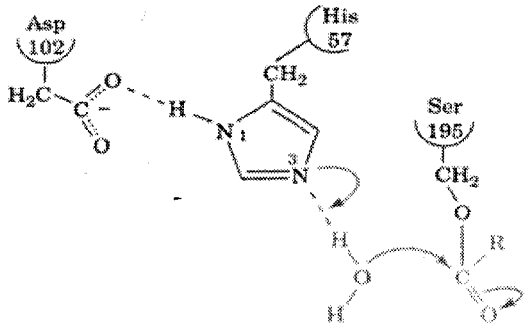

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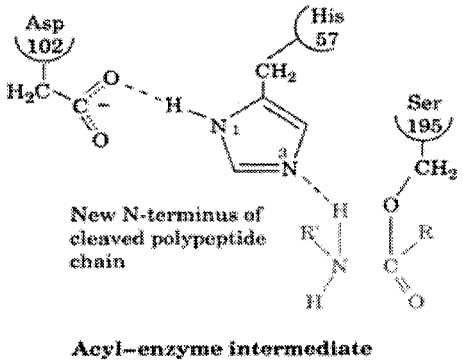

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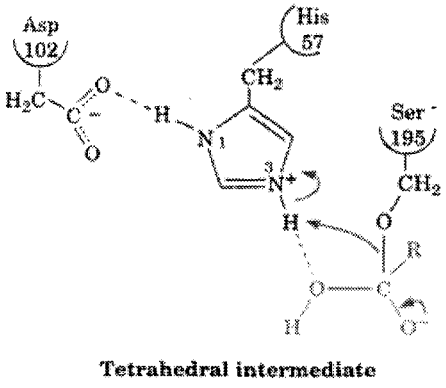

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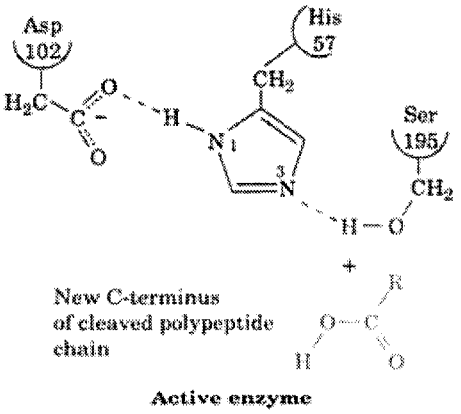


(4)

Figure 5. The catalytic mechanism of trypsin [1].

One synthetic substrate for trypsin is Nα-benzoyl-arginine-4-nitroanilide hydrochloride (BAPNA). An ideal chromogenic (color producing) substrate should be slightly soluble in aqueous solution, stable in the absence of the enzyme, sensitive to and specific for the enzyme under consideration, and capable of being cleaved upon hydrolysis. All these criteria are satisfied by BAPNA, which releases p-nitroaniline (yellow in color) upon exposure to trypsin [12]. The products of hydrolysis are Nα-benzoyl-arginine and p-nitroanailine (p-NA). Theoretically L-BAPNA is readily hydrolyzed by trypsin, whereas, D-BAPNA is a potent competitive inhibitor of trypsin [13]. Figure 6 shows the structure of L and D isomers of BAPNA.

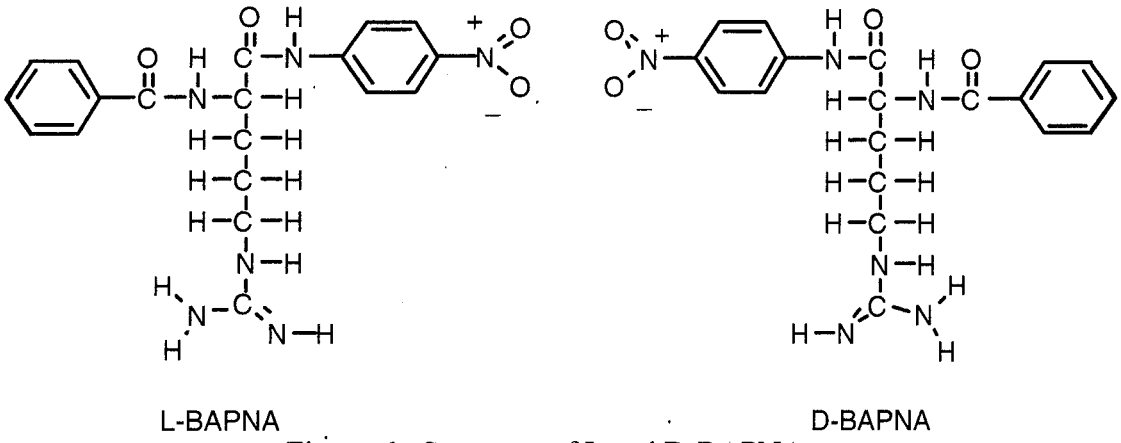


Figure 6. Structure of L and D-BAPNA.

Cations play a fundamental role in many biological functions. Calcium ions are specific for the activation of trypsinogen to trypsin and studies have shown that calcium ions can significantly enhance the catalytic efficiency of serine proteases during apoptosis (a natural form of cell death) [14, 15, 16]. Though it is well known that cations have a stabilizing effect on some serine proteases, the mechanism is not yet understood [16].

CHAPTER 2.0

LITERATURE REVIEW

Today there is an increasing interest in trypsin and its inhibitors due to their possible role in altering the nutritive value of food stuffs, their usefulness as models of protein-protein interactions, enzyme-substrate interactions, and their unique potential for clinical applications [18]. Research in this area has been going on at least since the 1900s, and many enzyme kinetic models have been developed since then [19]. A review of research reported in the open literature is provided.

2.1 Trypsin Kinetics

In the 1960s, Erlanger and coworkers synthesized two new chromogenic substrates for trypsin and estimated the trypsin activity colorimetrically using these chromogenic substrates. They generally used two assay methods: a) determination of kinetic constants by continuous measurement of the product of hydrolysis while the reaction proceeded in a Beckman DU spectrophotometric cuvette that was maintained at constant temperature; b) determination of pH optima by measuring the extent of hydrolysis after a fixed period of time [20]. They assumed that the hydrolysis of susceptible substrates by trypsin proceeds via a three-step mechanism

$$E + S \Leftrightarrow ES \qquad (1)$$

$$ES \Leftrightarrow ES' + P' \qquad (2)$$

$$ES' \Leftrightarrow E + P'' \qquad (3)$$

where ES is the Michaelis-Menten complex, E is the enzyme and P'' is the product. ES' and P' are intermediates. Figure 7 shows the rate of hydrolysis of DL- BAPNA as a function of trypsin concentration.

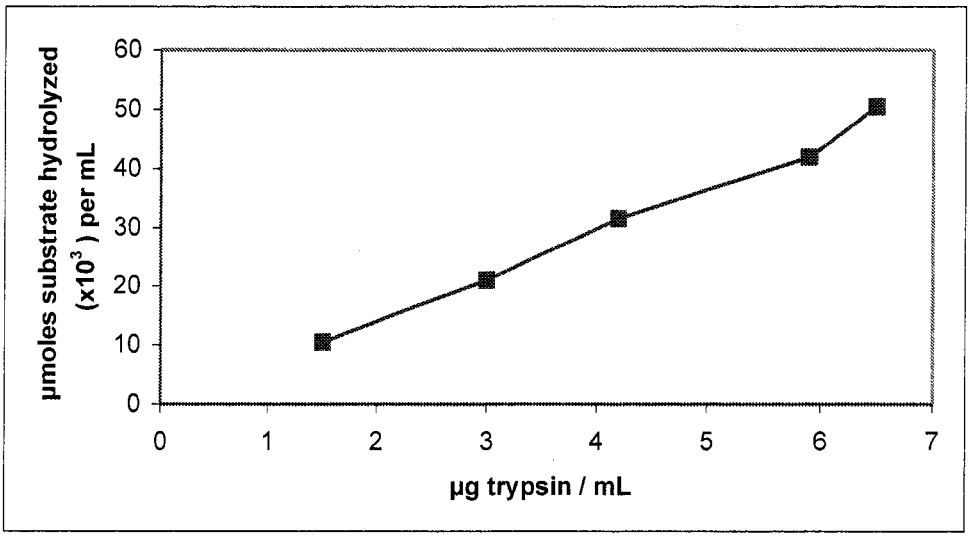


Figure 7. Rate of hydrolysis of DL-BAPNA as a function of trypsin concentration [18].

With the help of a Lineweaver-Burk plot, Erlanger and coworkers determined the values of the turnover number (k_{cat}) and Michaelis constant (K_M) to be 0.611 $(M{\cdot}S)^{-1}$ and 0.939 mM, respectively. In the late 1970s, Somorin et al. found the initial rate of trypsin-catalyzed hydrolysis of p-nitroanilide substrates by continuous measurement of free p-nitroaniline while the reaction proceeded in a cuvette inserted in a Hitachi 323 automatic spectrophotometer, as in method (a) of Erlanger et al. [12]. They determined the concentration of p-nitroaniline at 410 nm, the wavelength where the p-nitroanilide substrates have no contribution to the absorbance. Since their enzymatic process obeyed the Michaelis-Menten equation, they plotted the initial rate of trypsin hydrolysis versus substrate concentration using a Lineweaver-Burk plot [5]. Stewart did automated

analyses of the activities of trypsin, chymotrypsin and their inhibitors in 1971 using a Standard Technicon Auto analyzer [1]. He found that at higher enzyme concentrations (40-60 μg/mL) and lower substrate concentration (0.125 mg/mL) the standard curves were not linear, although they were reproducible. Barns and Elmslie compared two spectrophotometric methods (amidase and esterase) and a titrimetric method (esterase) for estimating trypsin in human duodenal fluid. Their results indicated that any of the three procedures was suitable for determining relative changes in trypsin levels although only the amidase gave a measure of the total tryptic activity present [21].

Recent kinetic studies by Adam Lesner and coworkers were performed using a Cary 3E spectrophotometer to determine p-nitroanilide substrates. The increase in absorbency at 410 nm resulting from p-nitroaniline release was measured as a function of time. Kinetic parameters such as k_{cat}, K_M and the specificity constant (k_{cat} / K_M) for each substrate were determined based on the equation

$$\frac{V}{E_T} = \frac{k_{cat}}{\left(1 + \left(\frac{K_M}{S}\right)\right)} \qquad \text{Equation 2}$$

where V is the velocity, E_T is the enzyme concentration and S is the substrate concentration. Their results are shown in Table 1 [22].

Table 1. Physiochemical properties of substrates studied and their kinetic parameters with bovine β-trypsin [22].

Substrate: Y-Ala-X-Abu-Pro-Lys-pNA	MW Calc. (found)	Rt (HPLC) min	K_M mM	k_{cat} s^{-1}	k_{cat} / K_M $M^{-1}s^{-1}$
Ac-Ala-Gly-Abu-Pro-Lys-pNA	618.7 (620)	13.6	338.0	43.0	127219.0
Ac-Ala-Ala-Abu-Pro-Lys-pNA	632.8 (634)	14.0	66.5	10.3	154887.3
H-Ala-Ala-Abu-Pro-Lys-pNA	590.7 (592)	12.6	238.8	13.8	57983.3
Ac-Ala-Abu-Abu-Pro-Lys-pNA	646.7 (648)	15.5	28.9	10.5	363321.8
Ac-Ala-Val-Abu-Pro-Lys-pNA	660.4 (662)	15.7	17.6	19.0	1079545.5
H-Ala-Val-Abu-Pro-Lys-pNA	618 (619.3)	9.1	28.7	9.6	334494.7
Ac-Ala-Ile-Abu-Pro-Lys-pNA	674.8 (676)	16.1	32.3	17.3	535604.8
H-Ala-Ile-Abu-Pro-Lys-pNA	632.7 (634)	15.2	16.9	32.0	189349.1
Ac-Ala-Phe-Abu-Pro-Lys-pNA	708 (709.4)	14.4	41.1	15.1	367397.2
H-Ala-Phe-Abu-Pro-Lys-pNA	666 (668.1)	11.8	157.1	23.0	156278.0
Ac-Ala-Glu-Abu-Pro-Lys-pNA	689.7 (691)	14.4	21.3	299.0	140675.3
Ac-Ala-Ser-Abu-Pro-Lys-pNA	648.7 (651)	14.3	29.4	377.3	128231.1

In the last decade, numerous studies revealed that enzymes could function as catalysts in non-aqueous solvents containing little or no water. For these reasons, strong efforts were made by Castro to determine the enzymatic activities of proteases dissolved in organic solvents. Kinetic analysis was performed using a Hitachi U-3110, UV-VIS spectrophotometer, and samples were analyzed by high-performance liquid chromatography (HPLC). The enzymatic activity of subtilisin (one of the three main

serine proteases) was studied using different solvents, and glycerol was selected as a solvent to compare kinetic parameters (V_{max} and K_M) with water for other proteases such as trypsin, pepsin, chymotrypsin, etc. The V_{max} and K_M values for trypsin in glycerol and water that they found are shown in Table 2 [23].

Table 2. The V_{max} and K_M values for trypsin in glycerol and water [23].

Solvent	V_{max} (μM/s·mg)	K_M (mM)
Water	0.46	2.7
Glycerol	0.17	25.0

2.2 Temperature and pH Used for Trypsin Kinetics

The pH activity curve for tryptic hydrolysis was studied by Erlanger and coworkers using two different chromogenic substrates (L-LPA and DL-BAPNA). They found that the optimal activity for hydrolysis of the arginine derivative (DL-BAPNA) occurred near pH 8.1 and that of L-LPA was approximately pH 9.25. For hydrolysis of DL- BAPNA, they used a Tris buffer with 0.042 M Tris, pH 8.2 containing 0.017 M $CaCl_2$. They performed this hydrolysis at room temperature (25 °C) and an incubation time of approximately 600 sec. The pH activity curves for the tryptic hydrolysis of DL- BAPNA and L- LPA are shown in Figure 8 [20]. BAPNA and BAPA denote the same substrate abbreviation.

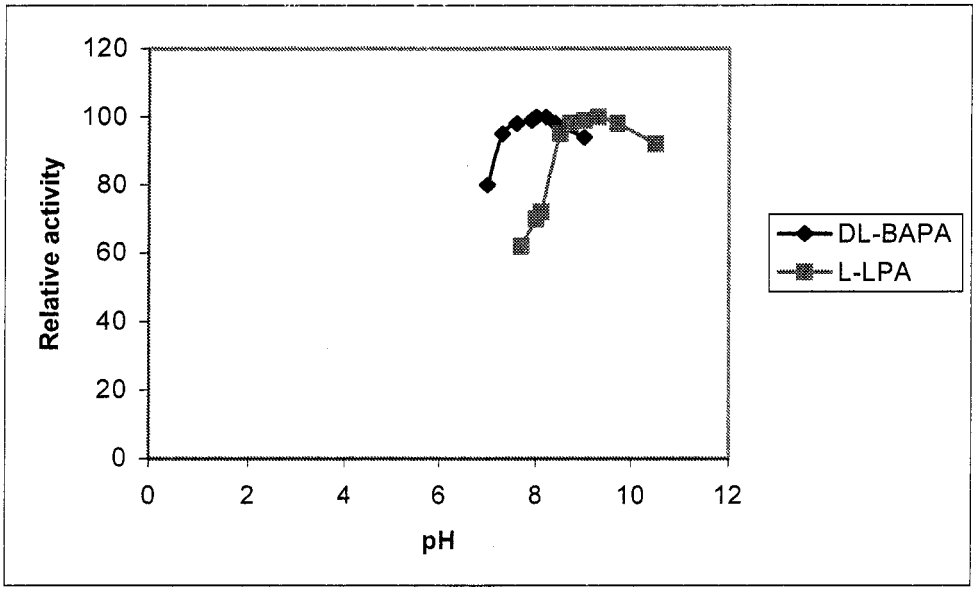


Figure 8. The pH activity curves for tryptic hydrolysis of DL-BAPNA and L-LPA [20].

Somorin et al. performed a similar experiment for trypsin hydrolysis using synthetic chromogenic arginine substrates. They used the 50 mM Tris-HCl buffer (pH 8.2) containing 20 mM $CaCl_2$. The enzyme reaction was carried out at 25 °C for 5 min [21]. Recent studies done by Adam Lesner and coworkers illustrate that they performed their experiments using a 0.1 M Tris-HCl (pH 8.3) buffer at 25 °C [5]. Compton and Fink studied the low temperature reactions of trypsin with p-nitroanilide substrates. Studies were conducted in the 0 to –30 °C temperature regions, over a range of pH values and using aqueous dimethyl sulphoxide as a cryosolvent. They found that at alkaline pH and –30 °C, the catalytic reaction appeared as a slow "burst" of product (or intermediate) followed by a turnover. The effect of temperature on the amplitude of burst in the reaction of trypsin with the substrate ZargpNA (N-carbobenzoxy-L-arginine-p-nitroanilide) is shown in Figure 9.

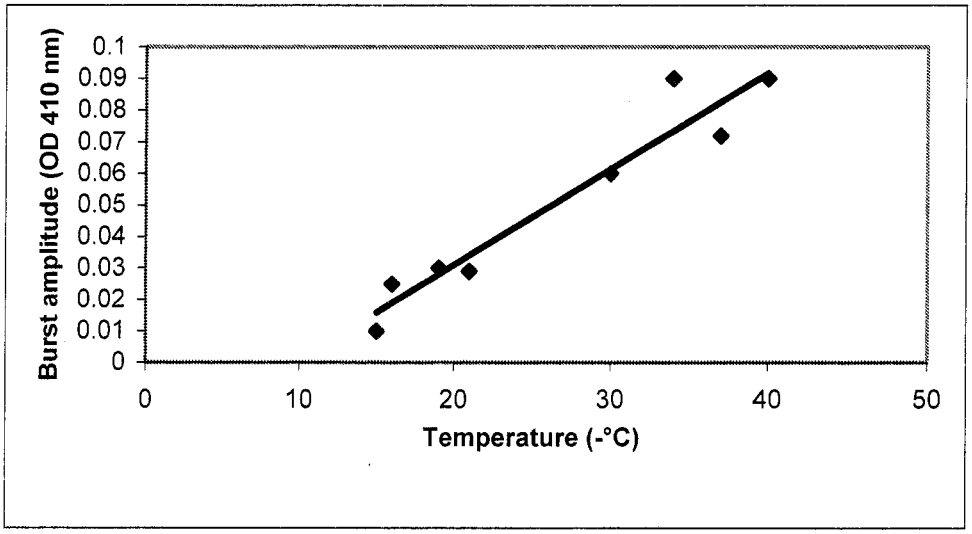


Figure 9. Effect of temperature on the reaction of trypsin with ZargpNA [4].

2.3 Substrates for Trypsin

Two new chromogenic substrates (L-lysine p-nitroanilide dihydrobromide (L-LPA) and benzoyl DL-arginine p-nitroanilide hydrochloride (DL-BAPNA) were prepared by Erlanger and coworkers. They found that the DL-BAPNA was more readily hydrolyzed by trypsin than L- LPA. Their data indicated that DL-BAPNA could be used in the systems containing as little as 1 μg trypsin/mL. In this respect, they regarded DL- BAPNA as the most sensitive amide substrate for trypsin. DL-BAPNA was proved to be useful in the manual assays for trypsin due to the selectivity of the enzyme for the substrate and the low blank values obtained in the absence of the enzyme. However, a similar study of the kinetics of the DL- BAPNA-trypsin reaction conducted by them presented some problems. They found that the L isomer of BAPNA was susceptible to enzymatic hydrolysis, whereas, the D isomer proved to be a competitive inhibitor [20]. Stewart performed the automated analyses of the activity of trypsin using BAPNA as

substrate. He used L- BAPNA as the substrate for his experiments since DL- BAPNA had problems with solubility and precipitated in the tubing and coils of the Auto analyzer [18]. Somorin and coworkers studied the action of trypsin on synthetic chromogenic arginine substrates. It was found that N-benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-arginine-p-nitroanilide hydrochloride (ZPVAPA·HCl) was split by trypsin more readily than N-benzyloxycarbonyl-L- arginine-p-nitroanilide hydrocloride (L-ZAPA·HCl), L- BAPNA· HCl, N-tosyl-L-arginine-p-nitro-anilide hydrocloride (L-TAPA·HCl) and DL- BAPNA·HCl, by factors of 100, 400, 600 and 1200, respectively. It was found that low concentrations of dimethyl formamide (DMF) enhanced the trypsin-catalysed hydrolyses of L-TAPA·HCl and L-ZAPA·HCl. Relative rates of trypsin-catalyzed hydrolyses of various arginine-p-nitroanilides are shown in Table 3 [5].

Table 3. Relative rates of trypsin-catalyzed hydrolysis of various arginine-p-nitroanilides [5].

Substrate	Relative rate
DL-BAPA.HCl	1.0
L-AAPA.HCl	9.0
L-BAPA.HCl	3.4
L-TAPA.HCl	1.8
L-ZAPA.HCl	10.0
L-BPVAPA.HCl	1177.0
L-ZPVAPA.HCl	1200.0

2.3 Effect of Cations on Trypsin Kinetics

Sipos and Merkel studied the effect of calcium ions on the activity and structure of trypsin. The experimental data on the optical rotatory dispersion of trypsin in the presence of calcium ions with respect to temperature at pH 7.8 suggested an increase in the helical content and some alteration in the β structure of the enzyme. Based on the studies done by Sage and Fasman, they concluded that the function of calcium during the temperature–dependent action was to maintain a specific compact conformation of enzyme molecules that is necessary for their catalytic activity [15].

Adebodun and coworkers investigated elevated levels of Ca (II) during apoptosis, a natural form of cell death involved in many physiological and pathological processes. Results of their studies indicated that Ca (II) can significantly enhance the catalytic efficiency of serine proteases and Ca (II) ions were also found to significantly protect the enzyme from inhibition by PMSF (phenylmethanesulfunylfluoride). Further, they studied the effect of other cations, such as Mg (II), K (I) and Na (I), on the microbial serine protease, subtilisin, but these cations did not seem to produce any significant rate enhancement. The range of concentrations of metal ions used in the pre-incubation of the stock enzyme was 0.1-60 mM [14]. Figure 10 shows the effect of Ca (II) on the activity of trypsin. Trypsin activity increases with the increase in calcium concentration.

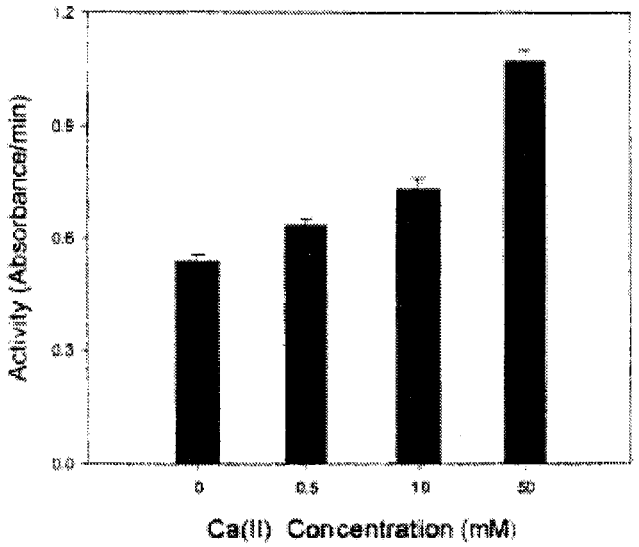


Figure 10. Effect of Ca (II) on the activity of trypsin [14].

The effects of calcium ions on the conformation and catalytic activity of trypsin were studied by Kotorman et al. in aqueous ethanol and water. Trypsin could retain 40% of its original activity in 85% ethanol at pH 3, but in the presence of 0.6 M Ca (II) in 85% ethanol, an increase in the activity of trypsin was observed relative to the level of activity detected in water. The near-UV CD spectra (250-300 nm) were monitored in order to obtain information on the tertiary structure of trypsin. The results show that the increase in stability of trypsin in aqueous ethanol in the presence of Ca (II) was due to an increased helical content and formation of a less compact overall structure in the presence of calcium ions [14]. This contradicts the results suggested by Sipos and Merkel. The near UV-CD spectra of trypsin in water and in ethanol, without and with Ca (II) is shown in Figure 11 [14].

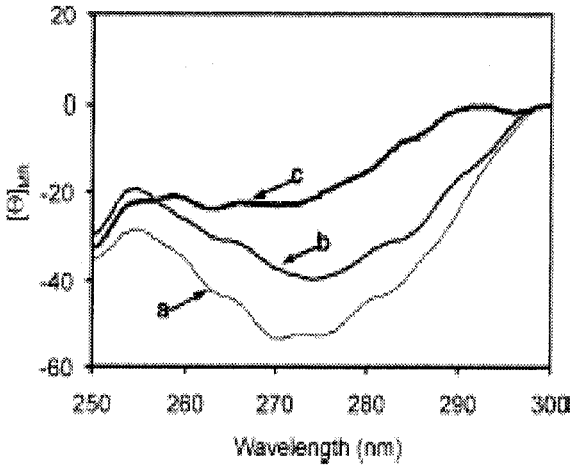


Figure 11. The Near – UV CD spectra of trypsin in water (a) and in 85% ethanol without (b) and with 0.6 M Ca (II) (c) [14].

A recent study done by Fashui et al. shows that Ce (III) at lower concentrations (0.5-5.0 μmol/L) could activate trypsin, but the effect of Ce (III) on the secondary structure of the enzyme was modest. They have also compared cerium with the other two metal ions, Cd (II) and Hg (II), and concluded that Ce (III) had a better activity for trypsin than that of Cd (II) and Hg (II) in vivo, and that Ce (III) can stimulate metabolism at lower concentrations [25].

2.5 Summary

BAPNA is a well-studied chromogenic substrate for trypsin. It releases p-nitroaniline (yellow in color) on exposure to trypsin. The values for k_{cat} and K_M for tryptic hydrolysis of L-BAPNA substrate were 0.611 $(M \cdot S)^{-1}$ and 0.939 mM, respectively [12]. The optimum pH was 8.0 and the temperature used was around 25°C.

Calcium ions promote the formation of active trypsin from trypsinogen. The stabilizing effect of calcium ions on serine proteases is well documented, but the effect of other cations on trypsin activity is not well studied. The range of cation concentration used was 0.1- 60 mM. Cerium ions seem to promote trypsin's activity at lower concentrations (0.5-5.0 μmol/L) but do not affect its secondary structure [25].

CHAPTER 3.0

RESEARCH HYPOTHESIS AND OBJECTIVE

3.1 Research Hypothesis

The hypotheses underlying this research are that there is an optimum pH for trypsin activity and that the addition of cations can change the activity of trypsin for DL-BAPNA substrate.

3.2 Research Objectives

- Measure the effect of pH and the cations Ca (II), Mg (II), K (I), Mn (II), Cr (III) and Ce (III) on the trypsin activity with DL-BAPNA as the substrate.
- Use non-linear regression and the resulting statistics to determine the kinetic parameters k_{cat} and K_M.
- Determine the metal ion-trypsin circular dichorism spectrum in order to detect any changes in conformation of trypsin in the presence of metal ions.

CHAPTER 4.0

EXPERIMENTAL METHODS AND MATERIALS

The trypsin activity was determined by evaluating the kinetic parameters k_{cat} and K_M. These parameters were determined by fitting the experimental rate versus substrate data to the Michaelis-Menten model. The experimental rate data was obtained from the product versus time data where the product concentration was measured using a HP 8452A diode array UV-VIS Spectrophotometer at 410 nm. In order to confirm the effect of cations on trypsin, the metal ion-trypsin circular dichorism (CD) spectrum was recorded at room temperature by an AVIV circular dichorism spectrometer (Model 215) with a quartz sample cell of 1 mm optical path length.

4.1. Experimental Matrix

Table 4 shows the experimental matrix used to determine the effect of DL-BAPNA substrate on trypsin activity at pH 7.8. This experiment was performed twice on different days for testing the reproducibility of the results.

Table 4. Experimental matrix to determine the effect of pH on trypsin activity.

Run No.	DL-BAPNA [S], mM	No. of samples.
1	2.00	2
2	1.75	2
3	1.50	2
4	1.00	2
5	0.750	2
6	0.500	2
7	0.250	2

- This matrix was repeated for pH 8.40, 9.00, 10.00 and 6.00. The optimum pH for trypsin activity was selected and used for further experiments.

The following experiments were done to test the effect of cations on trypsin activity. An experimental matrix is shown in Table 5.

Table 5. Experimental matrix to test the effect of cations on trypsin kinetics.

Run No.	No. of sample	DL-BAPNA [S], mM	Ca (II) mM	Mn (II) mM	K (I) mM	Mg (II) mM	Cr (III) mM	Ce (III) mM
1	2	2.00	0.005	0	0	0	0	0
2	2	2.00	0.5	0	0	0	0	0
3	2	2.00	10	0	0	0	0	0
4	2	2.00	0	0.005	0	0	0	0
5	2	2.00	0	0.5	0	0	0	0
6	2	2.00	0	10	0	0	0	0
7	2	2.00	0	0	0.005	0	0	0
8	2	2.00	0	0	0.5	0	0	0
9	2	2.00	0	0	10	0	0	0
10	2	2.00	0	0	0	0.005	0	0
11	2	2.00	0	0	0	0.5	0	0
12	2	2.00	0	0	0	10	0	0
13	2	2.00	0	0	0	0	0.005	0
14	2	2.00	0	0	0	0	0.5	0
15	2	2.00	0	0	0	0	10	0
16	2	2.00	0	0	0	0	0	0.005
17	2	2.00	0	0	0	0	0	0.5
18	2	2.00	0	0	0	0	0	10

- This matrix was repeated for 1.75, 1.50, 1.00, 0.750, 0.500 and 0.250 mM of DL-BAPNA.
- This matrix was run twice on different days to test reproducibility.

4.2 Reagents and Materials

The following reagents and materials were used in the experiment

- Buffer: 40 mM Tris-HCl, pH 7.8 at 25 °C.
- Enzyme solution: 0.1 mg/mL trypsin in 1 mM HCl (for UV-VIS measurements).
- Enzyme solution: 10 mg/mL trypsin in 1 mM HCl (for CD spectrum analysis).
- Substrate: 43.5 mg DL-BAPNA/mL dimethyl sulfoxide (DMSO).
- 1.50 mL disposable cuvettes (1.00 cm pathlength, Fisher Scientific Corporation).

Trypsin, DL-BAPNA and DMSO were purchased from Sigma Aldrich. $CaCl_2$, $MnCl_2$, $MgCl_2$, KCl, $CeCl_3$ and $CrCl_3$ were obtained from Fisher Scientific.

Equipment

The following equipments were used in the experiment

- HP 8452A Diode Array UV-VIS Spectrophotometer.
- AVIV Circular Dichorism Spectrometer (Model 215).

4.3 Sample Preparation

4.3.1 Preparation of Buffer

The buffer contains 40 mM Tris-HCl, pH 7.8 at 25 °C. The pH of the Tris buffer is sensitive to changes in temperature, thus it is important to adjust the pH at the temperature at which the buffer was used. The reactions were done at room temperature, which ranges from 24-25 °C. The buffer was prepared by dissolving 4.8496 grams of Trizma-base in approximately 950 mL deionized water, adjusting the pH at 25 °C to 7.8 with concentrated HCl, and diluting to a final volume of 1 liter with deionized water. In

order to determine the effect of cations on trypsin kinetics, the buffer should contain the required concentration of the selected cation.

4.3.2 Preparation of Substrate

Trypsin activity was determined by measuring the initial rate of reaction using a range of substrate concentrations from 0.250 to 2.00 mM. It was found that BAPNA had some solubility problems at concentrations over 2.00 mM-substrate concentration [16]. The stock substrate solution of 0.1 M concentration contains 43.5 mg BAPNA/mL dimethyl sulfoxide (DMSO). The substrate was completely dissolved in the DMSO with no crystals visible when the solution was mixed with the buffer. Since the substrate was dissolved in DMSO, pure DMSO was added to some samples to make the DMSO concentration constant in all samples. The volume of buffer, 0.1 M substrate solution, and pure DMSO needed for each tube is shown in Table 6. Samples are placed in a warming block at 37 °C to ensure that precipitation of the substrate does not occur. An aliquot of 900 μL of each sample was taken from the tube and transferred to a disposable cuvette.

4.3.3 Preparation of Enzyme Solution

Trypsin was dissolved in cold 1 mM HCl at a concentration of 0.1 mg/mL and 10 mg/mL and kept on ice until ready to use. The solution was mixed gently until completely dissolved. For UV-VIS measurements, 100 μL of 0.1 mg/mL enzyme solution was mixed with the substrate sample. The sample preparation matrix for

UV-VIS measurements are shown in Table 6. For CD measurements, the stock solution of trypsin (10 mg/mL) was diluted with 40 mM tris–HCl buffer containing the required cation concentration. The sample preparation matrix for CD measurements is shown in Table 7.

Table 6. Sample preparation matrix for UV-Vis measurements.

Substrate Solution			Sample Aliquot Volume, μL	Enzyme Solution [0.1 mg/mL] Volume, μL	Resulting substrate concentration DL-BAPNA [S], mM
Volume of DMSO, μL	Volume of 0.1 M [S] in DMSO, μL	Volume of Buffer, mL			
50.0	50.0	2.15	900	100	2.00
56.0	44.0	2.15	900	100	1.75
62.0	38.0	2.15	900	100	1.50
75.0	25.0	2.15	900	100	1.00
81.0	19.0	2.15	900	100	0.750
87.5	12.5	2.15	900	100	0.500
93.7	6.30	2.15	900	100	0.250

Table 7. Sample preparation matrix for CD measurements.

Sample No.	Enzyme solution (10 mg/mL) Volume, μL	Concentration of salt in 480 μL of 40 mM Tris-HCl buffer.
1	0	No salt (Blank)
2	20.0	No salt
3	20.0	0.005 mM $CaCl_2$
4	20.0	0.5 mM $CaCl_2$
5	20.0	0.005 mM $CrCl_3$
6	20.0	0.5 mM $CeCl_3$
7	20.0	0.005 mM $CeCl_3$

4.4 Experimental Set up

UV-Vis spectrophotometer

1. Seven numbered tubes that have tightly sealed caps were set up in a rack. Micropipettes were used to pipette the indicated volume of buffer, DMSO and 0.1 M substrate in DMSO into each tube.

2. The rack of tubes, the tube containing enzyme (on ice), cuvettes, wash bottle, soft wipes, waste beakers for solids (tips) and liquid were taken on a cart and moved near to the spectrophotometer.
3. The spectrophotometer was set up to read in kinetics mode at 410 nm using a run time of 120 seconds and a cycle time of 10 seconds.
4. Starting from the lowest concentration, an aliquot of 900 μL of the substrate solution was transferred to a disposable cuvette. The cuvette was placed in the holder in the spectrophotometer and immediately a blank reading (one without the enzyme) was taken. At 410 nm the absorbance due to substrate is negligible, but absorbance due to product is significant [18].
5. The cuvette was removed from the spectrophotometer, and 100 μL enzyme solution was added to it and mixed by drawing the sample into the micropipette several times. The sample was replaced in the spectrophotometer and was read in kinetics mode.
6. Absorbance versus time measurement was taken for each sample and saved as a "*.kd" file. A printout was taken when all the measurements were finished.
7. When the scan was done, the cuvette was removed, the solution was emptied into the waste beaker and the same procedures were repeated for the next sample.

CD Spectrometer

1. The first step in using the CD instrument is to turn on the nitrogen gas line and purge nitrogen gas for 15-30 minutes.

2. The power switch to the lamp was turned on. After a few seconds, when the "LAMP READY" light turns on, the red "PUSH TO START" button should be pushed to light the lamp. It is necessary to wait for 20 minutes for the lamp to warm up before starting the experiments.

3. The power switch to the "CPU and Instrument" was turned on. The Aviv data collection program was started. The main control panel would appear.

4. Under the menu choices, "Experiment configuration" was selected to set the parameters for the experiment such as wavelength (200-260 nm) and temperature (25 °C).

5. From the menu choice "Exit/Save Configuration" was chosen. The main AVIV program menu would appear.

6. The sample was placed in a 1 mm quartz sample cell. The cell along with a Peltier type cell holder was placed in the sample compartment with the narrow side facing the operator.

7. From the menu, "RUN EXPERIMENT" was selected and the data was collected.

8. Each spectrum collected should be given a different name and saved to the disk or hard drive.

9. In order to convert raw CD data (in millidegrees) to quality graphs, the background (blank) was subtracted from each scan in the AVIV software using the math operations menu.

10. The scans were then saved as text files in the data browser window. The extra text that follows the two columns of data in each text file was removed and saved.

11. Each text file was imported to an Excel spreadsheet and a graph was plotted using the "XY (Scatter)" option in the Excel chart wizard.

12. The same procedure was repeated for each sample.

4.5 Analysis

According to Beer's law, the amount of light that a solution absorbs is directly proportional to the concentration of solute. However, there are limitations to spectrophotometers. Spectrophotometers often display a non-linear response at high absorption levels because of stray light. Most spectrometers are useful for measuring absorbances up to 1 [6]. A hypothetical Beer's law plot for absorbance (A) versus concentration for a spectrophotometer is shown in Figure 12.

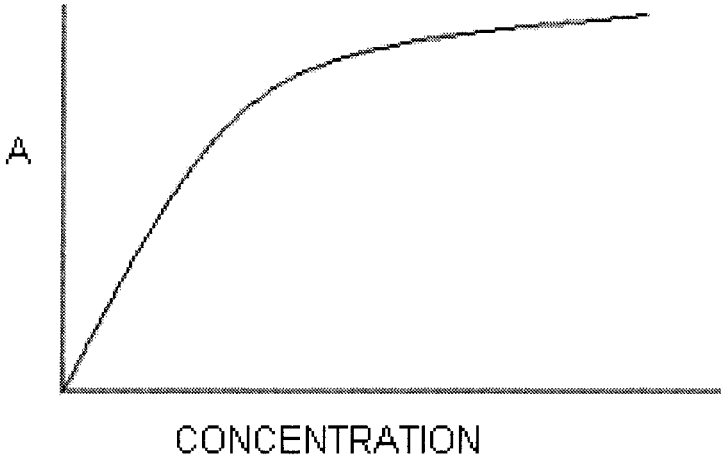


Figure 12. Beer's law plot for absorbance versus concentration.

The linear portion of the curve is sensitive to concentration changes. Therefore, only the linear portion of each absorbance curve obtained from the spectrophotometer is used to obtain the product concentration required to calculate the rate of the reaction (V). From the standard calibration curve, the product concentration (p-nitroaniline) can be obtained from the corresponding absorbance reading from the spectrophotometer. In order to obtain a standard curve, a stock solution of 0.1 mM p-nitroaniline was prepared in the Tris-HCl (40 mM) buffer of pH 7.80 at 25 °C. The Tris buffer was used as the diluting medium and the blank. A series of 6 samples was prepared for a standard absorbance curve from 0.10 mM to 0.005 mM p-NA. The absorbance was recorded in a 1.00 cm path length cuvette from 300 to 500 nm, setting the notation at 410 nm. A printout was taken for absorbance versus wavelength curves and the absorbance for each concentration at 410 nm. Table 8 shows the absorbance as a function of p-NA concentration at a wavelength of 410 nm.

Table 8. Absorbance as a function of p-NA concentration at a wavelength of 410 nm.

p-NA solution Concentration (mM)	Absorbance (AU) at 410 nm
0.0050	0.0712
0.010	0.0771
0.025	0.1217
0.050	0.4271
0.075	0.6478
0.10	0.8145

The calibration curve for the product at pH 7.80 is shown in Figure 13.

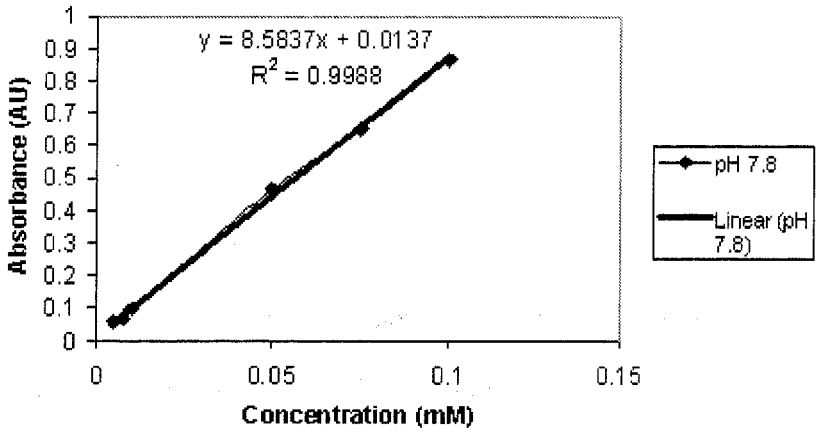


Figure 13. The calibration curve for pH 7.80.

Reaction rate calculation

Considering a constant volume batch reactor, the material balance will be

In - out ± generation = accumulation Equation 3

For a reactant, the batch reactor design equation is given by the formula

$$V = \frac{-dC}{dt}$$ Equation 4

where $\frac{dC}{dt}$ is the rate of change of substrate concentration and V is the rate expression given by the Michaelis-Menten equation shown in Equation 1.

Since there is a one to one stochiometry between substrate and product we can use a product design equation to calculate the rate using the formula,

$$V = \frac{dP}{dt}$$ Equation 5

where P is product concentration and t is the time. The linear portion of P versus t data was used to determine ΔP and Δt to calculate the rate of the reaction. The corresponding substrate concentration [S] was used in the Michaelis-Menten equation.

A non-linear optimization program on Polymath was used to determine whether the rate and substrate concentration data fit into Michaelis-Menten kinetics and provide the values of K_M and V_{max} (maximum rate) directly. Non-linear regression determines the values of parameters for an equation, whose form should be specified, that cause the equation to best fit a set of data values. The quality of fit was analyzed from the statistics (95% confidence, residuals, correlation coefficient (R^2) and variance).

k_{cat} (the catalytic efficiency of the enzyme) was calculated using the formula

$$k_{cat} = \frac{V_{max}}{E_T} \qquad \text{Equation 6}$$

where E_T is the total or the initial enzyme concentration. The k_{cat} value denotes the activity of the enzyme, and K_M value suggests the enzyme's affinity for the substrate. The K_M is unique for each enzyme- substrate pair on an assumption that E_T is a constant [1]. This procedure is also used to determine the effect of cations on the activity of trypsin where the concentrations of cations vary from 0.005-10 mM.

The total enzyme concentration, E_T, was determined using the Beer's law equation,

$$A = E_T \varepsilon l \qquad \text{Equation 7}$$

where A is the absorbance of trypsin at 280 nm, ε ,the extinction coefficient of trypsin at 280 nm, which is 14.3 $mM^{-1}cm^{-1}$(per Sigma product information), l, the path length which is 1.00 cm and E_T, the total enzyme concentration.

CHAPTER 5.0

RESULTS AND DISCUSSION

5.1. Effect of pH on Trypsin Activity

The effect of pH on trypsin activity was calculated the same for each experimental run. The procedure is described in detail using the pH 7.80 run as an example.

Absorbance versus time data for seven different substrate concentrations was obtained from the spectrophotometer. A sample absorbance versus time data for 2.00 mM substrate concentration is shown in Figure 14.

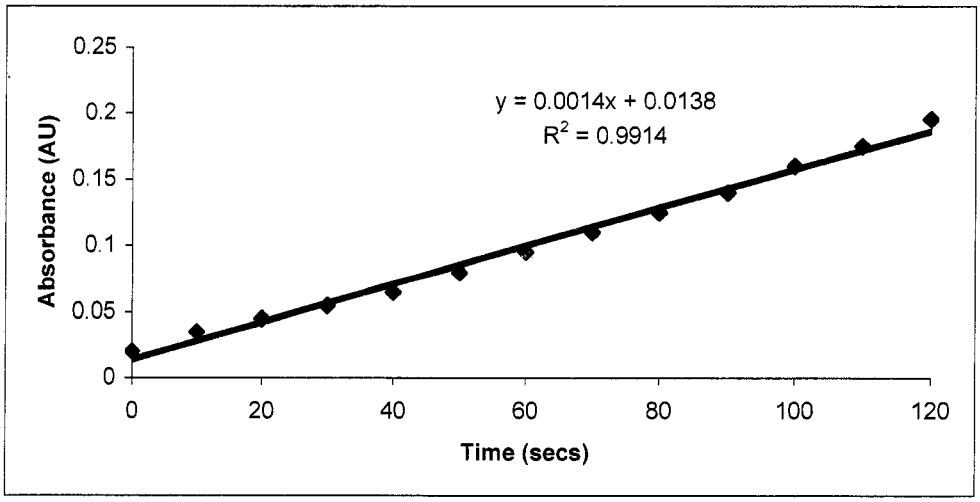


Figure 14. Absorbance versus time data for 2.00 mM substrate concentration.

The absorbance was always below 0.3 due to low product formation. Therefore, only the lower part of the calibration curve (absorbance below 0.3) was used to determine the rate values, as shown in Figure 15.

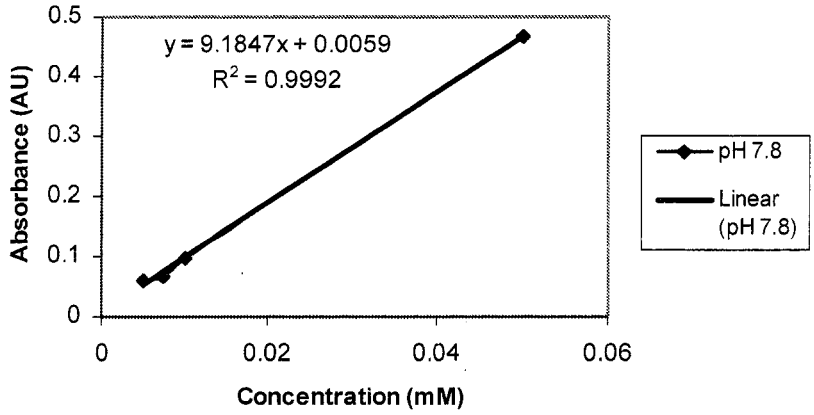


Figure15. The standard absorbance curve for the product at pH 7.80.

The product concentration corresponding to the absorbance is obtained from the product calibration graph at pH 7.80. The product concentration versus time plot is shown in Figure 16. A linear fit was confirmed by examination of the correlation coefficient, R^2 (0.9992).

The calibration graph is more linear in the absorbance range 0.3–0.9 as shown in Figure 13. Therefore, future experiments should consider increasing the product concentration in order to work in the absorbance range 0.3-0.9. One way of increasing the product concentration is to increase the amount of trypsin or substrate appropriately so that the resulting absorbances are in 0.3-0.9 range, which is a less error-prone region of the calibration curve.

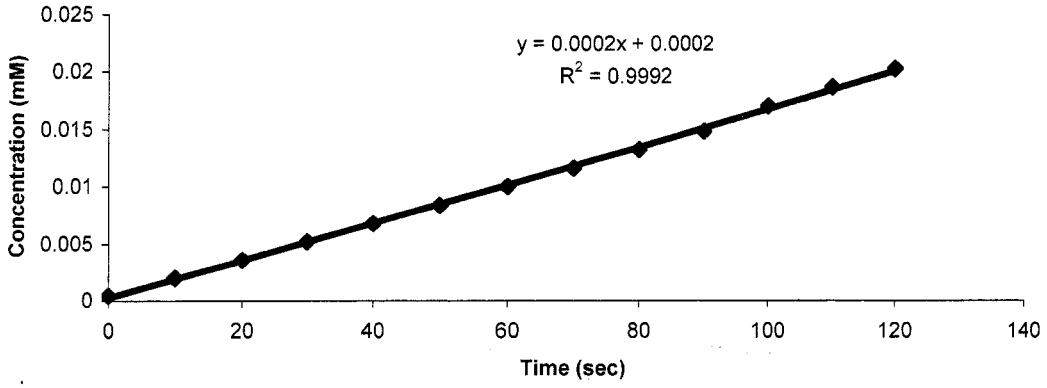


Figure 16. Product concentration versus time plot for 2.00 mM substrate concentration.

Since the results are linear, the difference in product concentration divided by the difference in time gives the rate of the reaction (V). From equation 5,

$$V = \frac{dP}{dt}$$

where dP = (Product concentration corresponding to absorbance at 100 sec)

- (Product concentration corresponding to absorbance at 20 sec)

and dt = difference in time = (100-20) sec.

Table 9 shows the reaction rate data for the original sample.

Table 9. Reaction rate data for the original sample.

Substrate Concentration (mM)	Absorbance at 100 sec (AU) A1	Concentration corresponding to A1 (mM) C1 (x 10^2)	Absorbance at 20 sec (AU) A2	Concentration corresponding to A2 (mM) C2 (x 10^3)	Rate (V) $=\frac{(C1-C2)}{80}$ (mM/sec) (x 10^4)
0.250	0.070	0.700	0.034	3.1	0.48
0.500	0.145	1.50	0.065	6.4	1.1
0.750	0.105	1.10	0.030	2.6	1.1
1.00	0.120	1.20	0.030	2.6	1.2
1.50	0.160	1.70	0.050	4.8	1.5
1.75	0.180	1.90	0.050	4.8	1.8
2.00	0.160	1.70	0.045	4.3	1.6

A non-linear optimization program on Polymath is used to determine whether the rate and substrate concentration data fit into Michaelis-Menten kinetics and provide the values of K_M and V_{max} (maximum rate) directly. In the Polymath results "V_{max}" represents the maximum rate and the "K_M" represents the Michaelis –Menten constant. Polymath results are shown below.

POLYMATH Results

Nonlinear regression (L-M)

Model: $R = V_{max}*S/(K_M + S)$

Variable	Ini guess	Value	95% confidence
V_{max}	1	2.498E-04	8.989E-05
K_M	1	0.7830213	0.7043164

Nonlinear regression settings
Max # iterations = 64

Precision

R^2	= 0.910849
R^2adj	= 0.8930188
Rmsd	= 4.804E-06
Variance	= 2.261E-10

General

Sample size	= 7
# Model vars	= 2
# Indep vars	= 1
# Iterations	= 9

In order for the regression results to be statistically valid, the confidence intervals (95% confidence) must be smaller than the respective parameter values (in absolute values), R^2 (correlation coefficient) value should be more than 0.90, the variance should be small and the residuals should be random. Since all these criteria are satisfied by the Polymath results shown above, the rate versus substrate concentration data fits the Michaelis-Menten model. Only four out of all the experimental values had a very low R^2 value and the 95% confidence was higher than the respective parameter values. There can be three reasons for the R^2 value to be low: (1) the model was not good (2) not enough data points to fit the parameters, K_M and V_{max} or (3) the data points were not appropriate. Since the Michaelis-Menten model is a well known model used for enzyme catalyzed reactions and there were enough (seven) data points to fit the two parameters, the only reason for the R^2 value to be low is the data points not being appropriate and therefore, the four values were eliminated based on this reason.

The pH activity curve for the tryptic hydrolysis of DL-BAPNA is shown in Figure 17. Trypsin does not show any activity at pH 6.02. The results show that the optimal activity for the hydrolysis of the DL-BAPNA substrate occurs near pH 7.80.

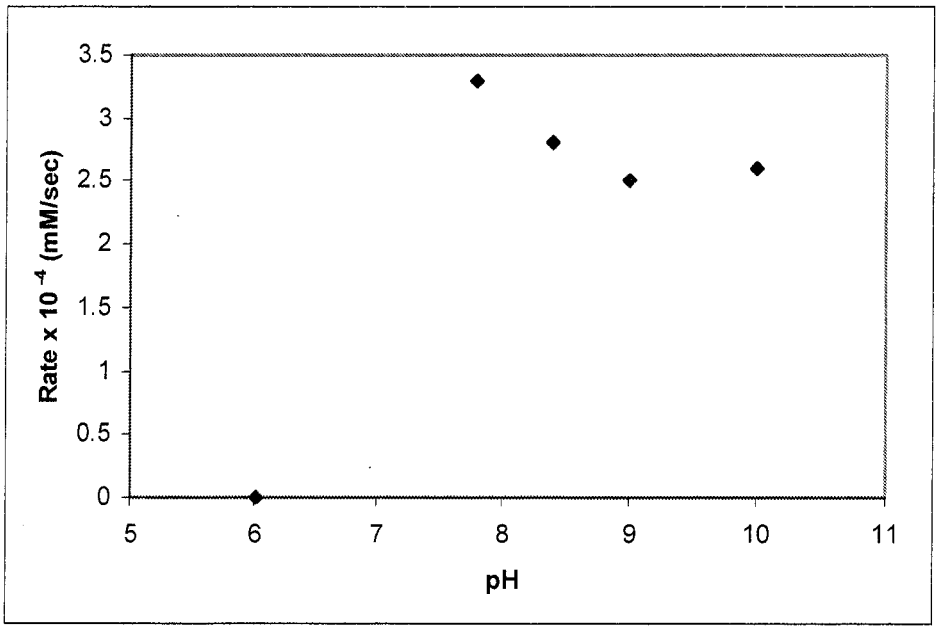


Figure 17. pH activity curve for the tryptic hydrolysis of DL-BAPNA.

Enzymes like trypsin contain a large number of acidic and basic groups, mainly situated on their surface. The charges on these groups will vary with the pH of their environment, according to their acid dissociation constants. The change in pH therefore affects the charge on the exterior surfaces of the enzyme, their net charge and the reactivity of the catalytically active groups in the active sites. These changes in charges with pH affect the activity and structural stability of the enzyme. The nucleophilic attack in the catalytic mechanism of trypsin involves transfer of a proton to the imidazole ring of histidine thereby forming an imidazolium ion [1]. This process is aided by the polarizing effect of the carboxylate ion of aspartate, which is hydrogen bonded to histidine. The decomposition of the tetrahedral intermediate involves a proton donation from N3 of histidine, which is a general base catalysis. The pKa for imidazoyl (histidine) and aspartate residue ranges from 6-9 and 4-9, respectively. Changes in pH can influence

the protonation state of these residues thereby affecting the overall mechanism of the reaction. Changes in pH may also change the charge properties of the substrate, DL-BAPNA, so that either the substrate cannot bind to the active site or it cannot undergo catalysis. The arginine residue in DL-BAPNA has a pKa range from 11-13. However, the change in pH from 6 to 10 does not affect the charge on the arginine residue and thus, does not have much effect on the substrate. At the pH optimum, the charges on the amino acids produce a favorable pattern of charges on the active site and the exterior surface of the enzyme thereby affecting the folding conformation that is necessary for speeding up the rate of the reaction.

Assuming the total enzyme concentration, E_T was the same for all the runs, E_T was determined using the Beer's law equation. By using a spectrophotometer, the absorbance of trypsin solution at 280 nm was found to be 0.13008 with 0.001 M HCl as the blank, as shown in Figure 18. The value of total enzyme concentration was calculated from equation 5 and was determined to be 0.0091 mM.

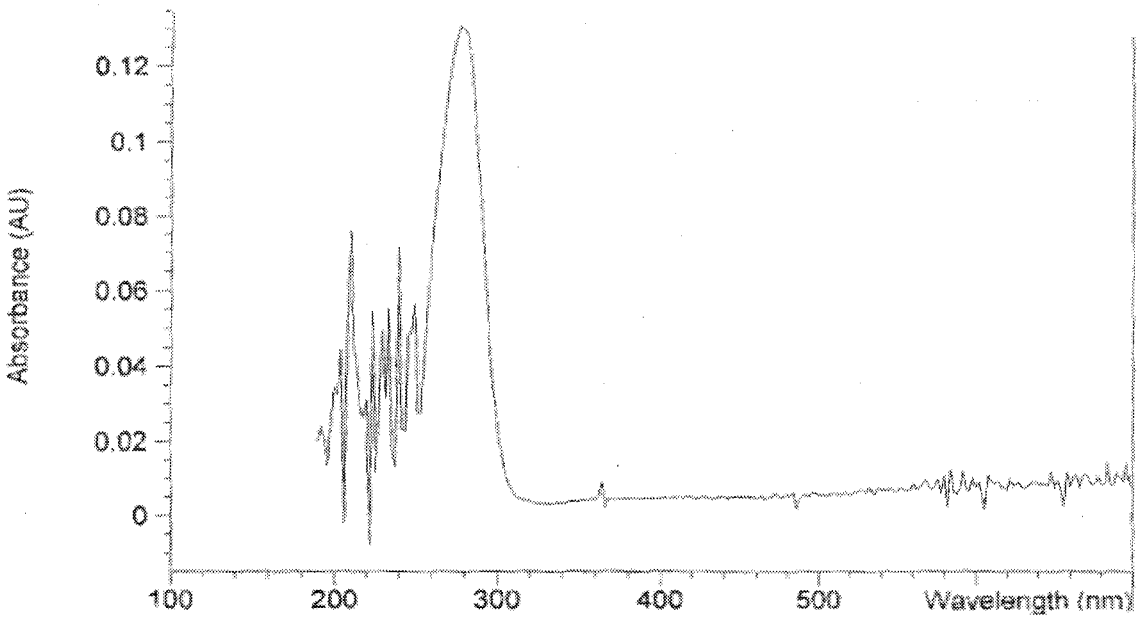


Figure 18. Absorbance of trypsin at 280 nm.

The k_{cat} value was determined using the equation 6 and was found to be 0.027 sec^{-1}. Similar calculations were done for the duplicate samples. The calculated values for the original and duplicate samples are shown in Table 10.

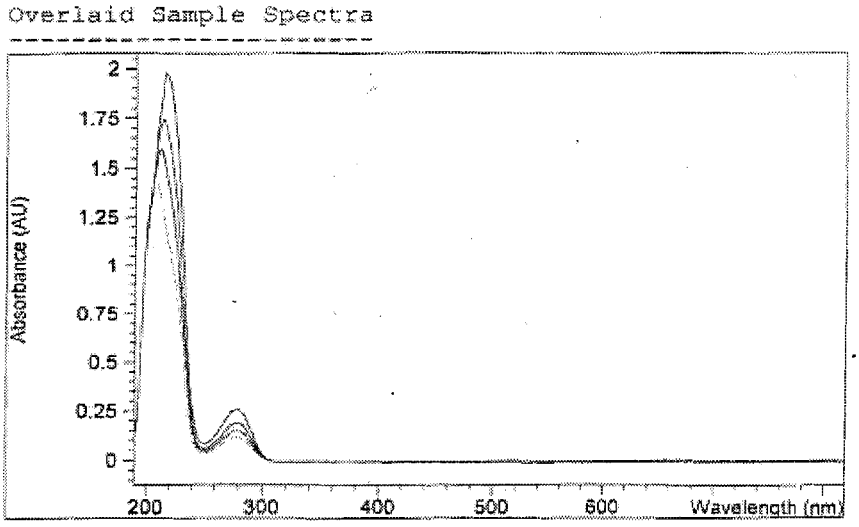
Table 10. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 7.80.

DL-BAPNA	Original sample	Duplicate 1	Duplicate 2	Duplicate 3	Standard deviation
K_M (mM)	0.78	0.54	2.1	1.5	±0.92
k_{cat} (sec^{-1})	0.027	0.025	0.049	0.040	±0.011

There was a significant deviation in the K_M and k_{cat} values for the original and duplicate samples as shown in Table 10. This was the case for all the experiments. Considering this situation, an experiment was done to test if the assumption that the

enzyme concentration was constant in all the runs was true since the K_M and k_{cat} value depends on the total or initial enzyme concentration. Five different samples containing 0.1 mg/ml trypsin solution were prepared and their absorbances were determined at 280 nm using a spectrophotometer in order to find out their total enzyme concentration using Beer's law equation.

Figure 19 shows the absorbance of five different samples containing 0.1 mg/ml of trypsin solution at 280 nm with 0.001 M HCl as the blank.



Sample/Result Table

#	Name	Abs<260nm>	#	Name	Abs<280nm>
1	sample1	0.25551	4	sample4	0.11739
2	sample2	0.15132	5	sample5	0.15323
3	sample3	0.18869			

Figure 19. Absorbance of five different samples containing 0.1 mg/ml enzyme solution at 280 nm.

As shown in Figure 19, there was a significant difference in the absorbance for each sample and hence the total enzyme concentration varied for each sample. Therefore, the assumption that the total enzyme concentration was constant in all the runs

proved to be wrong. This might explain the high standard deviation in the K_M and k_{cat} values for the original and duplicate samples for each experiment. Hence, future experiments should consider evaluating the total enzyme concentration for every experimental sample and also measure a higher amount of trypsin and dilute to the required concentration since the chances of making error is more when measuring only a very little amount of trypsin like 2 mg to prepare the required 0.1 mg/ml enzyme concentration for each experimental run.

5.2. Effect of Cations on Trypsin Activity

The effects of different concentrations of cations on the activity of trypsin were determined at pH 7.80. The enzyme activity measurements were performed at the cations concentration range of 0.005-10.0 mM. Calcium and cerium ions showed some effect on the activity of trypsin, while the cations, K (I), Mg (II), Mn (II) and Cr (III) produced no significant effect. Other cations tested, Zn (II), Fe (II) and Fe (III), precipitated in the buffer due to their low solubility product constants.

In Figures 20 and 21, the k_{cat} value with zero concentration of cation is the control. In Figure 20, considering the average value of k_{cat} for different concentrations of calcium, there was an increase in the activity of trypsin from 0.005 mM to 0.5 mM Ca (II) and then the activity decreased for 10.0 mM Ca (II) concentration. However, when considering the standard deviation represented by the error bars, the results are not statistically significant due to overlapping of the error bars.

As shown in Figure 21, the activity of trypsin increased from 0.005 to 0.5 mM $CeCl_3$ concentration, but at 10.0 mM $CeCl_3$ concentration, there was no activity for

trypsin. Since the error bars for 0.005 mM Ce (III) concentration overlap with the control, there was no statistically significant change in the trypsin activity for 0.005 mM Ce (III) concentration. The difference in activity is statistically significant at 0.5 M showing an increase of 66%.

Each sample was run four times. The standard deviation was more than 40%. It should be noted that the E_T was not measured for each run and thus, as discussed previously, probably contributes significant error to the results.

In order to find out the effect of cations on trypsin activity, the assays done in the literature pre-incubated the trypsin with appropriate concentrations of cation. Therefore, the results could not be compared with the literature since a different method was employed here. Future experiments can try employing the method used in the literature for finding the effect of cations on trypsin activity.

There can be two plausible explanations for the observed Ce (III) induced effect on trypsin's activity. Those factors are: (1) that the Ce (III) ions induce a conformational change of the enzyme to a form that is more catalytically efficient, or (2) that the cation forms a complex with suitable side chains in the active site to induce the activity without affecting the structure of the enzyme.

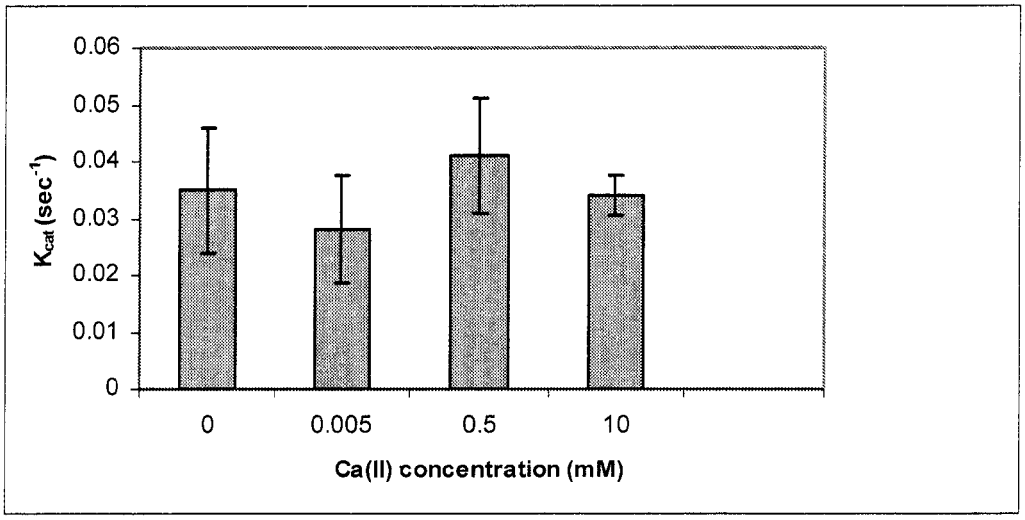


Figure 20. Calcium induced enhancement of trypsin enzyme.

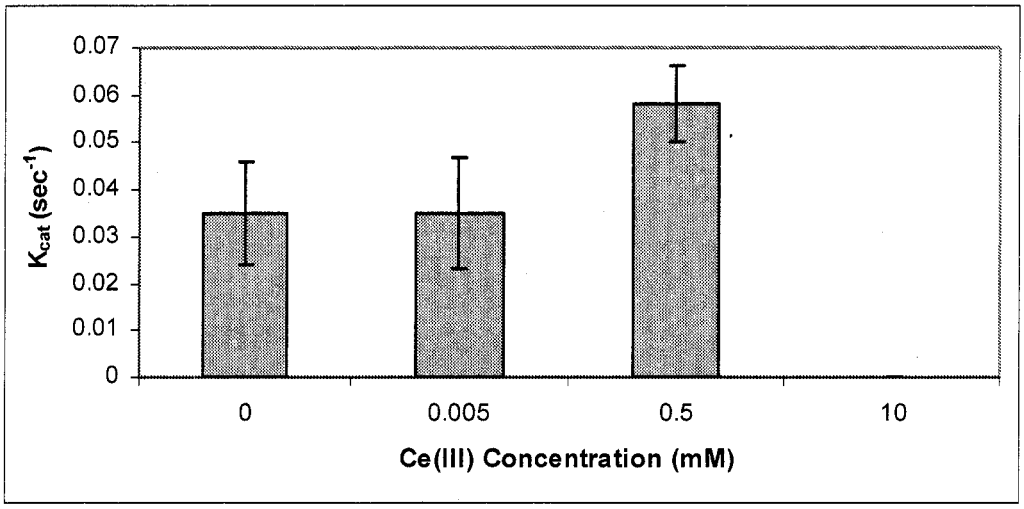


Figure 21. Effect of $CeCl_3$ concentration on the activity of trypsin.

When comparing cerium (III) and calcium (II), cerium had a better effect on the activity of trypsin than calcium. A comparison of average k_{cat} and K_M values for different concentrations of Ca (II) and Ce (III) is shown in Table 11.

Table 11. Comparison of average k_{cat} and K_M values for different concentrations of Ca (II) and Ce (III).

Cation Concentration	Ca (II) (mM)			Ce (III) (mM)	
	10.0	0.50	0.005	0.50	0.005
K_M (mM/Liter)	1.3	1.8	1.0	1.8	1.0
k_{cat} (sec^{-1})	0.034	0.041	0.026	0.058	0.035

5.3. Effect of Ce (III) and Ca (II) on the Secondary Structure of Trypsin

In order to examine the effect of Ce (III) and Ca (II) on the secondary structure of trypsin, the far ultraviolet CD spectra of trypsin treated with a buffer solution containing Ce (III) or Ca (II) were determined with trypsin without salt as the control.

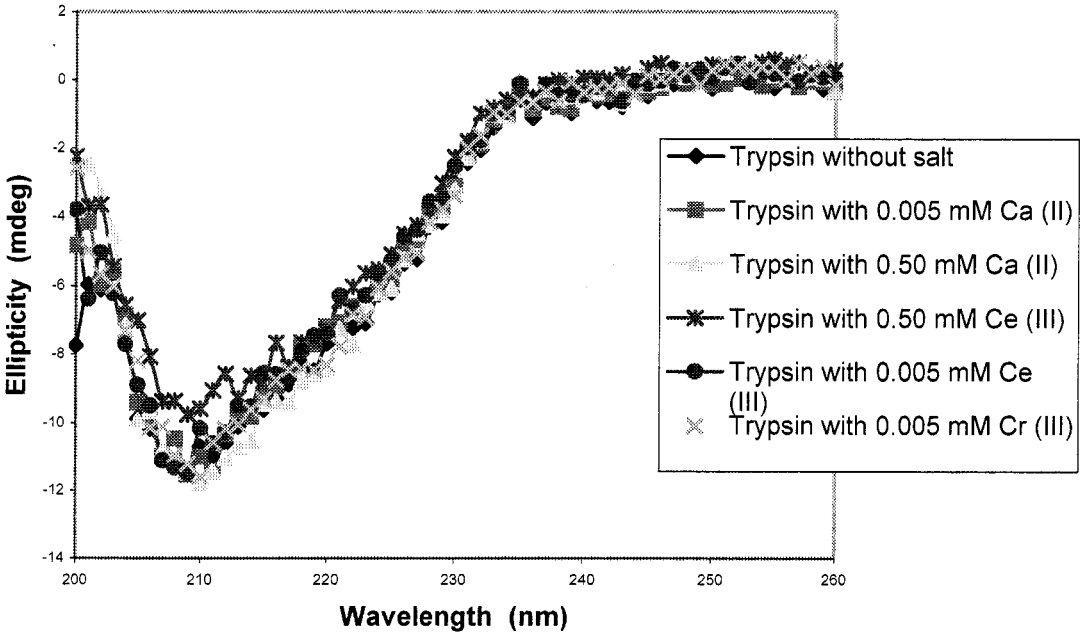


Figure 22. Effect of metal ions on the CD spectra of trypsin.

Figure 22 shows the effect of different concentrations of metal ions (Ce (III), Ca (II) and Cr (III)) on the CD spectra of trypsin. It can be seen that these spectra are essentially identical down to 200 nm. There is a slight difference between the CD spectra of trypsin with 0.5 mM $CeCl_3$ and that of the control but any slight variations are within the noise tolerance of the instrument. Though cerium and calcium ions affect the activity of trypsin in the assays, they do not seem to affect the secondary structure of trypsin. Figures 23 and 24 show different concentrations of calcium and cerium ion-trypsin dichroism spectra with the control, respectively.

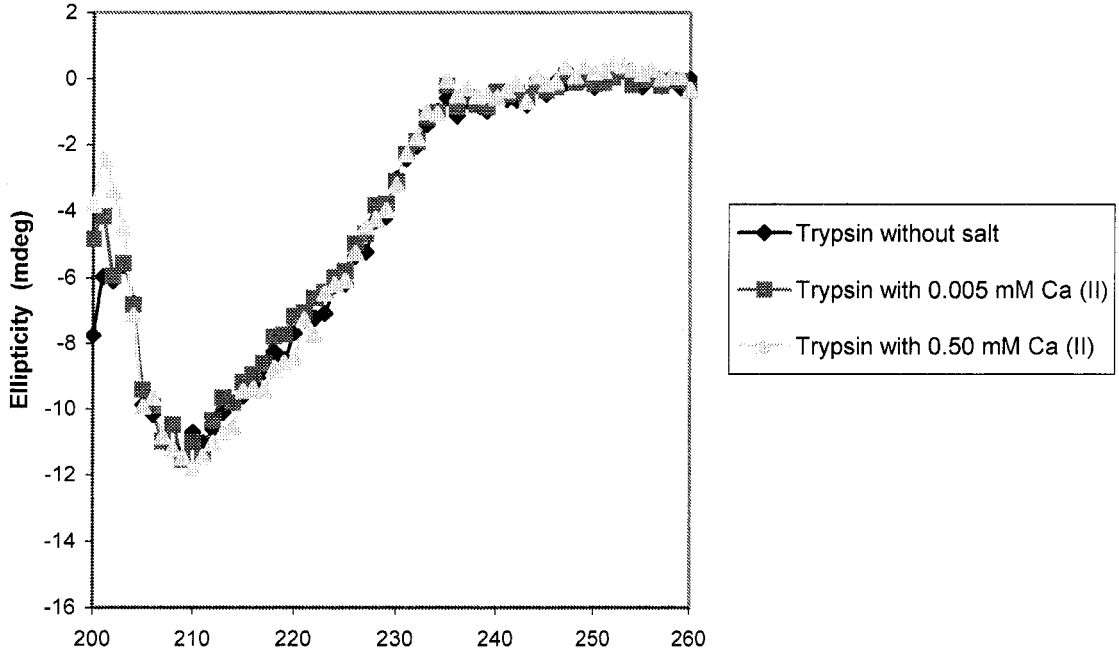


Figure 23. Effect of different concentrations of calcium on the CD spectrum of trypsin.

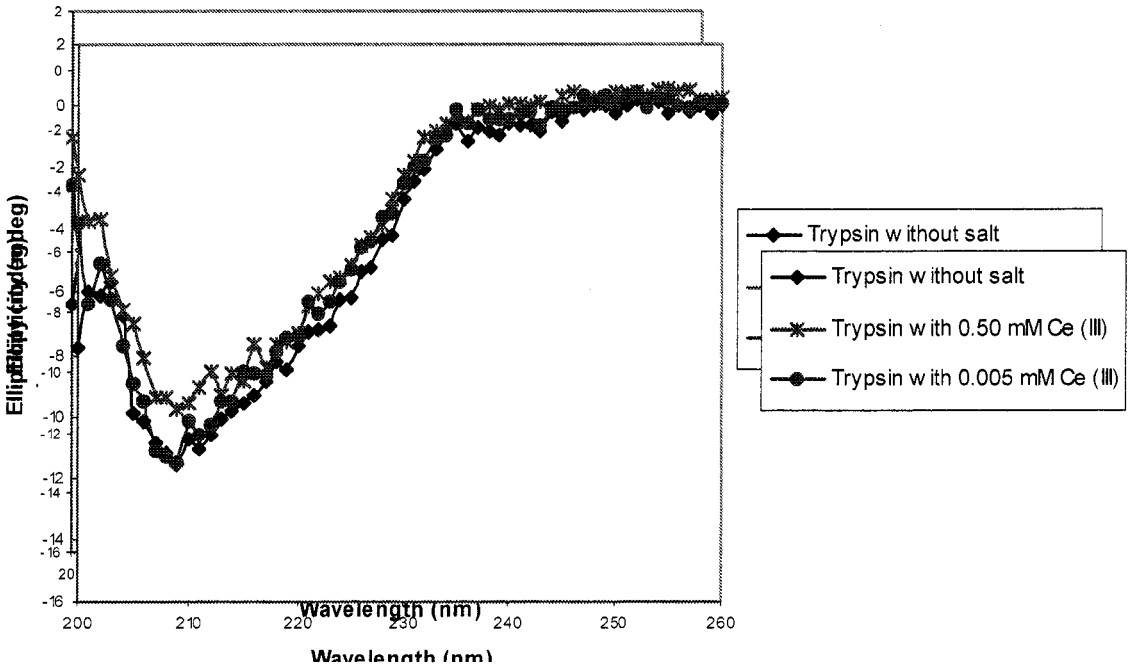


Figure 24. Effect of different concentrations of cerium ions on the CD spectra of trypsin.

This observation eliminates the hypothesis that the cations induce a significant change in the secondary structure of the enzyme. It is, therefore, more likely that the cations interact with the amino acids in the active site or induce a subtle conformational change of the enzyme in order to affect the activity of trypsin without affecting the secondary structure of the enzyme. The preferential binding of the transition state (or the tetrahedral intermediate) over the enzyme-substrate complex or the acyl-enzyme intermediate is responsible for much of the catalytic efficiency of serine proteases [1]. There is a possibility that the cations stabilize the negatively charged tetrahedral intermediate by electrostatic ion-pair interactions at the active site [1]. Also the charge density of the cations can influence the structure of the water molecules surrounding the enzyme. The changes in the water structure surrounding the enzyme can change the folding conformation of the enzyme thereby changing the catalytic activity of the enzyme

[26]. However, when comparing cerium with the other cations, it is more likely that the unique ratio of atomic size to charge of Ce (III) is preferred over the other cations for the observed effects. Hence, the unique charge to mass ratio of the cations can make them suitable to pair with suitable side chains in the active site or induce a subtle conformational change of the enzyme to affect the activity of the enzyme.

CHAPTER 6.0

CONCLUSIONS

The effect of cations and pH on trypsin kinetics was studied using an UV-VIS spectrophotometer. The enzyme kinetic parameters, k_{cat} and K_M, were determined in order to determine the effect of Ca (II), Mg (II), Ce (III), Mn (II), Cr (III) and K (I) on trypsin by initial-rate experiments. Trypsin has a maximum rate at pH 7.80. Among the ions, trypsin showed some activity in the presence of calcium and cerium. At 0.5 mM Ce (III) concentration, there was a 66% enhancement in the activity of trypsin. At concentrations greater than 10 mM, trypsin showed no activity in the presence of cerium. Though there was an increase in activity of trypsin for 0.5 M Ca (II), the results were not statistically significant due to overlapping of the error bars. Though Ce (III) and Ca (II) could affect the activity of trypsin in the assays, they did not alter the secondary structure of trypsin as shown by the CD spectra.

6.1 Future directions

The concentration of product formed was very low. Future experiments should consider increasing the trypsin or substrate concentration in order to increase the product concentration so that the absorbance is in a less error prone region of the calibration curve. In order to make the substrate more soluble, a higher (but not too high) amount of DMSO can be used. However, too high a concentration of DMSO can denature the enzyme. It is advisable to evaluate the total or initial trypsin concentration for each experimental run and measure a higher amount of trypsin and dilute to the required concentration. Further, pre-incubating the trypsin solution with required concentration of

cation can be examined for finding the effect of cations on trypsin activity. Also, future experiments could investigate the effect of anions on trypsin activity since the substrate and active site of trypsin consist of positively charged arginine and histidine residue, respectively.

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APPENDIX A- Experiments to find the effect of pH on trypsin activity.

Sample calculations for different pH runs are shown in this section.

1. pH 8.40, June 16, 2004

Absorbance versus time data for seven different substrate concentrations were obtained from the spectrophotometer. A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure A1. For each substrate concentration, the absorbance at 100 sec and 20 sec are noted down.

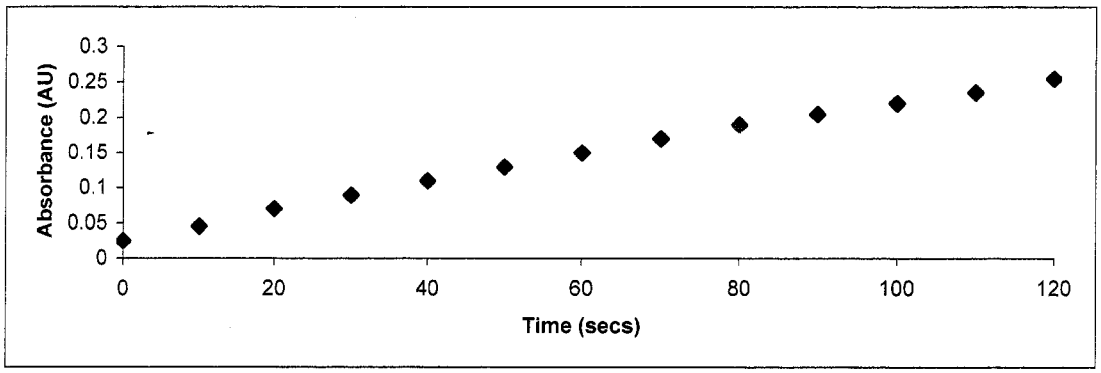

Figure A1. Absorbance versus time data for 2.00 mM substrate concentration.

The calibration curve for pH 8.40 is shown in Figure A2. The product concentration corresponding to the absorbance is obtained from the calibration graph for pH 8.40.

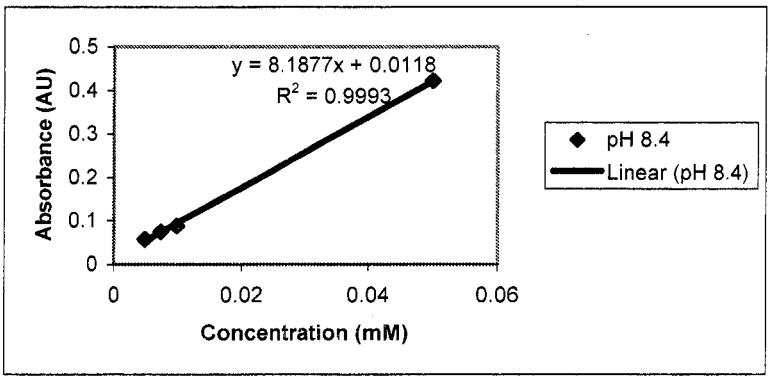

Figure A2. Calibration curve for pH 8.40.

The difference in product concentration divided by the time gives the rate of the reaction (V). From equation 5,

$$V = \frac{dP}{dt}$$

where dP = (Product concentration corresponding to absorbance at 100 sec)

- (Product concentration corresponding to absorbance at 20 sec)

and dt = difference in time = (100-20) sec.

Table A1 shows the reaction rate data for the original sample.

Table A1. Reaction rate data for the original sample.

Substrate Concentration (mM)	Absorbance at 100 sec (AU) A1	Concentration corresponding to A1 (mM) C1 (x 10^2)	Absorbance at 20 sec (AU) A2	Concentration corresponding to A2 (mM) C2 (x 10^3)	Rate (V) $=\frac{(C1-C2)}{80}$ (mM/sec) (x 10^4)
0.250	0.130	1.40	0.030	2.2	1.5
0.500	0.135	1.50	0.040	3.4	1.5
0.750	0.140	1.60	0.040	3.4	1.5
1.00	0.170	1.90	0.050	4.7	1.9
1.50	0.210	2.40	0.065	6.5	2.2
1.75	0.230	2.60	0.070	7.1	2.4
2.00	0.220	2.60	0.070	7.1	2.3

A non-linear optimization program on Polymath is used to determine whether the rate and substrate concentration data fit into Michaelis-Menten kinetics, and to provide the values of K_M and V_{max} (maximum rate) directly. In the Polymath results "V_{max}" represents the maximum rate and the "K_M" represents the Michaelis –Menten constant. Polymath results are shown below.

POLYMATH Results

Nonlinear regression (L-M)

Model: $R = V_{max}*S/(K_M + S)$

Variable	Ini guess	Value	95% confidence
Vmax	1	2.623E-04	7.972E-05
K_M	1	0.3070359	0.372552

Nonlinear regression settings
Max # iterations = 64

Precision
R^2 = 0.6717229
R^2adj = 0.6060675
Rmsd = 8.436E-06
Variance = 6.974E-10

General
Sample size = 7
Model vars = 2
Indep vars = 1
Iterations = 14

In order for the regression results to be statistically valid, the confidence intervals (95% confidence) must be smaller than the respective parameter values (in absolute values), R^2 value should be greater than 0.90, the variance should be small and the residuals should be random. Since all these criteria are satisfied by the Polymath results shown above, the rate versus substrate concentration data fit the Michaelis-Menten model.

The value of total enzyme concentration was calculated from equation 5 and was determined to be 0.0091 mM. The k_{cat} value was determined using the equation 6 and was found to be 0.028 sec^{-1}. Similar calculations were done for the duplicate samples. The calculated values for the original and the duplicate samples are shown in Table A2.

Table A2. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 8.40.

DL-BAPNA substrate	Original sample	Duplicate 1 sample	Duplicate 2 sample	Duplicate 3 sample	Standard Deviation
K_M (mM)	0.30	0.21	0.76	2.0	± 0.81
k_{cat} (sec^{-1})	0.028	0.029	0.032	0.036	± 0.0035

2. pH 9.00, June 17, 2004.

A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure A3. The calibration curve for pH 9.00 is shown in Figure A4.

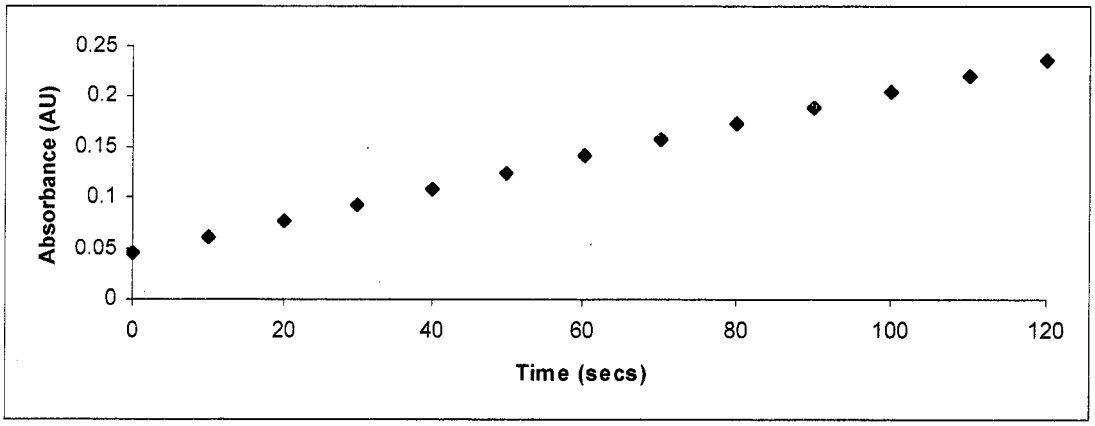

Figure A3. Absorbance versus time data for 2.00 mM substrate concentration.

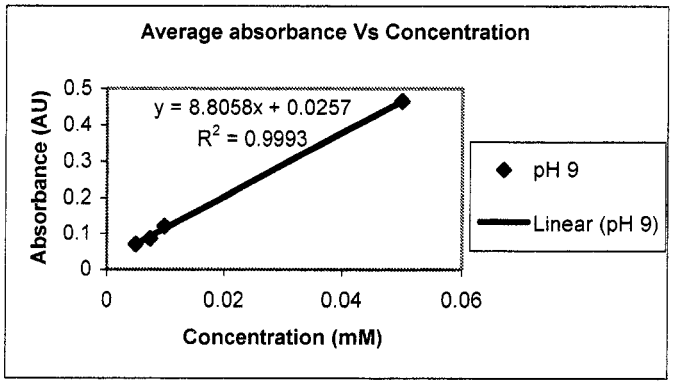

Figure A4. Calibration curve for pH 9.00.

Table A3 shows the reaction rate data for the original sample.

Table A3. Reaction rate data for the original sample.

Substrate Concentration (mM)	Rate (V) (mM/sec) (x 10^4)
0.500	0.83
0.750	1.1
1.00	1.4
1.50	1.7
1.75	1.7
2.00	1.9

POLYMATH Results

Nonlinear regression (L-M)

Model: $R = V_{max}*S/(K_M + S)$

Variable	Ini guess	Value	95% confidence
Vmax	1	3.054E-04	7.49E-05
K_M	1	1.342124	0.6615985

Nonlinear regression settings
Max # iterations = 64

Precision
R^2 = 0.9815744
R^2adj = 0.976968
Rmsd = 2.004E-06
Variance = 3.614E-11

General
Sample size = 6
Model vars = 2
Indep vars = 1
Iterations = 6

The k_{cat} value was determined using the equation 6 and was found to be 0.034 sec^{-1}. Similar calculations are done for the duplicate samples. The calculated values for the original and the duplicate samples are shown in Table A4.

Table A4. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 9.00.

DL-BAPNA substrate	Original sample	Duplicate 1 sample	Duplicate 2 sample	Duplicate 3 sample	Standard Deviation
K_M (mM)	1.2	1.3	0.57	0.32	± 0.48
k_{cat} (sec^{-1})	0.028	0.034	0.028	0.022	± 0.0049

3. pH 10.00, June 17, 2004.

A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure A5.

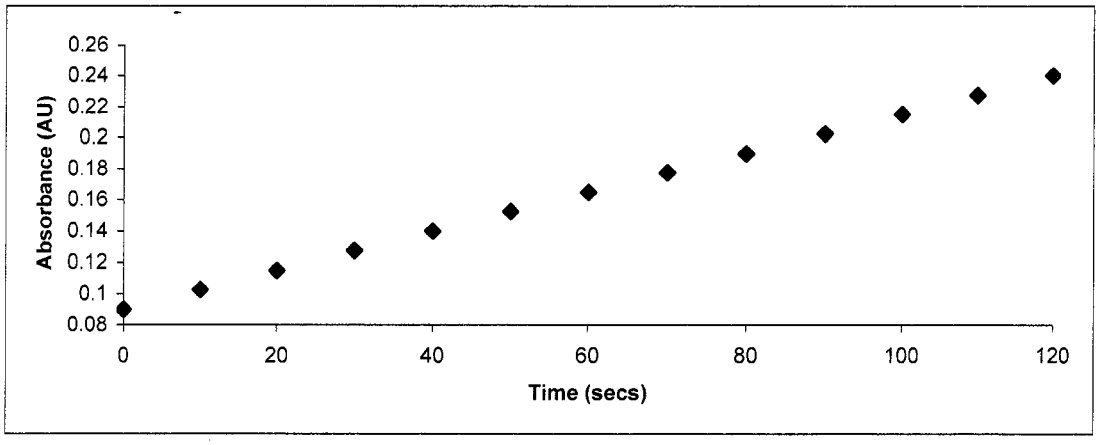

Figure A5. Absorbance versus time data for 2.00 mM substrate concentration.

The calibration curve for pH 10.00 is shown in Figure A6.

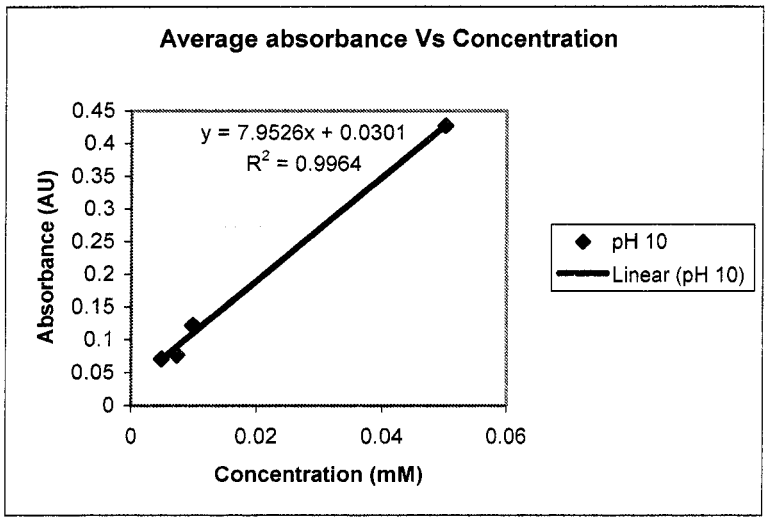

Figure A6. Calibration curve for pH 10.00.

Table A5 shows the reaction rate data for the original sample.

Table A5. Reaction rate data for the original sample.

Substrate Concentration (mM)	Rate (V) (mM/sec) (x 10^4)
0.250	0.45
0.500	0.60
0.750	1.2
1.00	1.4
1.50	1.8
1.75	1.9
2.00	1.7

POLYMATH Results

Nonlinear regression (L-M)

Model: $R = V_{max}*S/(K_M + S)$

Variable	Ini guess	Value	95% confidence
Vmax	1	3.291E-04	1.293E-08
K_M	1	1.4884776	1.11E-04

Nonlinear regression settings
Max # iterations = 64

Precision
R^2 = 0.9203807
R^2adj = 0.9044569
Rmsd = 5.637E-06
Variance = 3.114E-10

General
Sample size = 7
Model vars = 2
Indep vars = 1
Iterations = 8

The k_{cat} value was determined using the equation 6 and was found to be 0.036 sec^{-1}. Similar calculations are done for the duplicate samples. The calculated values for the original and duplicate samples are shown in Table A6.

Table A6. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 10.00.

DL-BAPNA substrate	Original sample	Duplicate 1 sample	Duplicate 2 sample	Duplicate 3 sample	Standard Deviation
K_M (mM)	1.49	0.37	0.34	2.23	± 0.92
k_{cat} (sec^{-1})	0.036	0.027	0.026	0.053	± 0.012

4. pH 6.02, June 16, 2004.

Trypsin does not show any activity at pH 6.02.

APPENDIX B - Experiments to find the effect of cations on trypsin activity.

The optimal activity for the hydrolysis of the DL-BAPNA substrate occurred near pH 7.80. Therefore, the effects of different concentrations of cations on the activity of trypsin were determined at pH 7.80.

1. 0.5 mM $CaCl_2$, July 15, 2004

A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure B1.

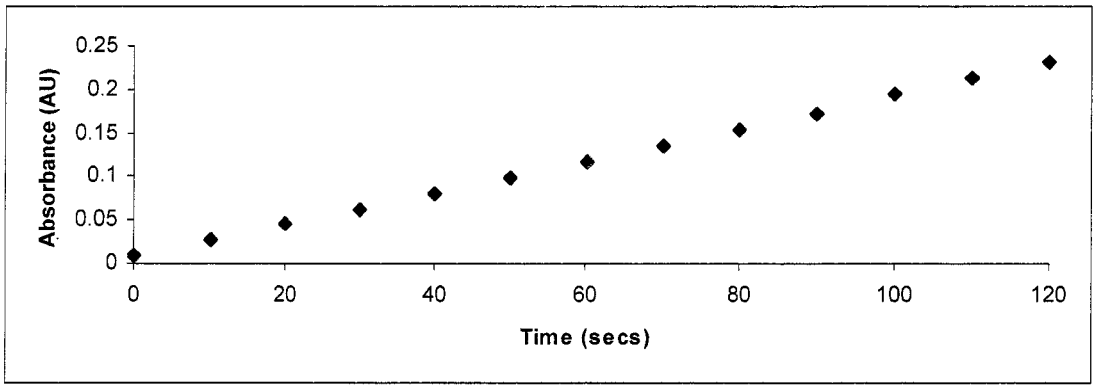

Figure B1. Absorbance versus time data for 2.00 mM substrate concentration.

Table B1 shows the reaction rate data for the original sample.

Table B1. Reaction rate data for the original sample.

Substrate Concentration (mM)	Rate (V) (mM/sec) (x 10^4)
0.250	0.37
0.500	0.96
1.00	1.3
1.50	1.8
1.75	2.0
2.00	1.9

POLYMATH Results

Nonlinear regression (L-M)

Model: Rori = Vmax*S/(Km+S)

Variable	Ini guess	Value	95% confidence
Vmax	1	3.643E-04	1.706E-04
Km	1	1.6353734	1.4412824

Nonlinear regression settings
Max # iterations = 64

Precision

R^2	= 0.9766941
R^2adj	= 0.9708676
Rmsd	= 3.604E-06
Variance	= 1.169E-10

General

Sample size	= 6
# Model vars	= 2
# Indep vars	= 1
# Iterations	= 8

The k_{cat} value was determined using the equation 6 and was found to be 0.040 sec^{-1}. Similar calculations are done for the duplicate samples. The calculated values for the original and duplicate samples are shown in Table B2.

Table B2. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 7.80.

DL-BAPNA substrate	Original sample	Duplicate 1 sample	Duplicate 2 sample	Duplicate 3 sample	Standard Deviation
K_M (mM)	1.6	2.7	1.8	0.94	± 0.73
k_{cat} (sec^{-1})	0.040	0.055	0.035	0.032	± 0.010

2. 50 mM $CaCl_2$, July 15, 2004

A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure B2.

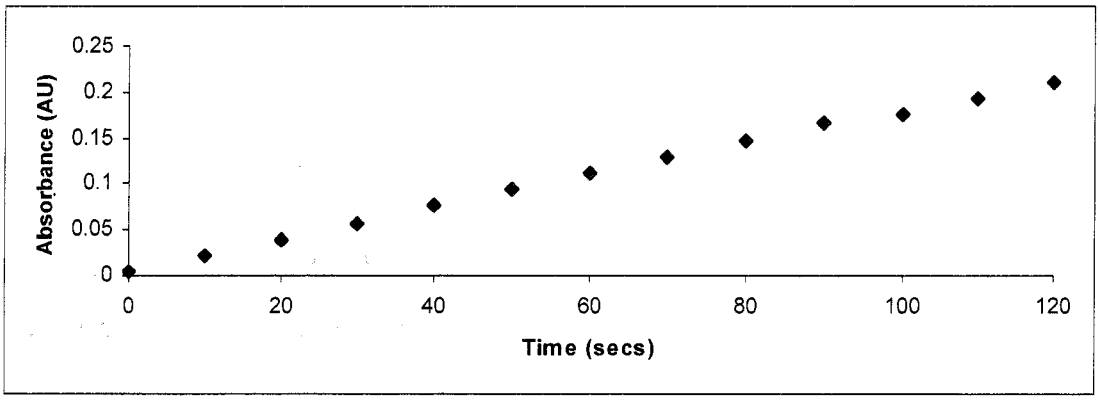

Figure B2. Absorbance versus time data for 2.00 mM substrate concentration.

Table B3 shows the reaction rate data for the original sample.

Table B3. Reaction rate data for the original sample.

Substrate Concentration (mM)	Rate (V) (mM/sec) (x 10^4)
0.250	0.43
0.500	1.0
0.750	1.2
1.00	1.4
1.50	1.8
1.75	1.9
2.00	2.0

POLYMATH Results
Nonlinear regression (L-M)

Model: Rori = Vmax*S/(Km+S)

Variable	Ini guess	Value	95% confidence
Vmax	1	3.335E-04	7.56E-05
Km	1	1.3370643	0.6000404

Nonlinear regression settings
Max # iterations = 64

Precision
R^2 = 0.9859842
R^2adj = 0.983181
Rmsd = 2.31E-06
Variance = 5.228E-11

General
Sample size = 7
Model vars = 2
Indep vars = 1
Iterations = 6

The k_{cat} value was determined using the equation 6 and was found to be 0.037 sec^{-1}. Similar calculations are done for the duplicate samples. The three duplicate samples were eliminated due to low R^2 value. The calculated values for the original and duplicate samples are shown in Table B4.

Table B4. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 7.80.

DL-BAPNA substrate	Original sample	Duplicate 1 sample	Duplicate 2 sample	Duplicate 3 sample	Standard Deviation
K_M (mM)	1.3	0.61	0.45	0.20	± 0.47
k_{cat} (sec^{-1})	0.037	0.026	0.019	0.015	± 0.0096

3. 0.5 mM $CeCl_3$, July 21, 2004

A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure B3.

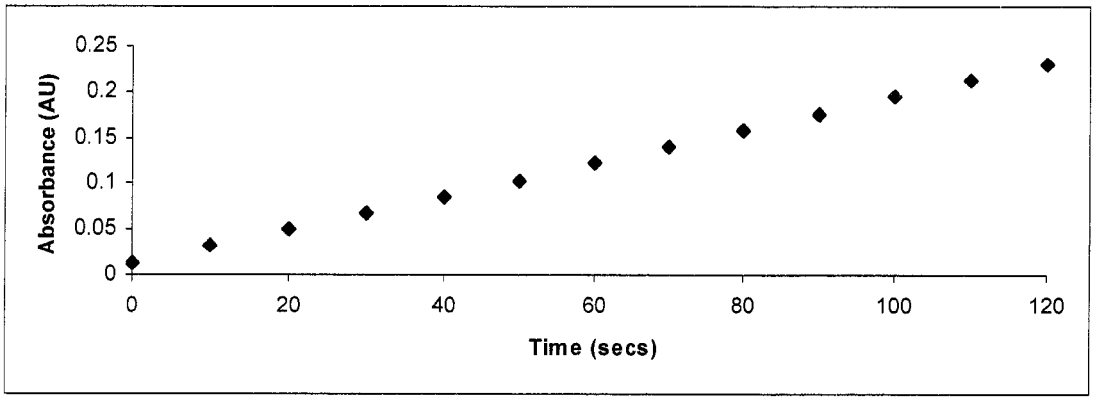

Figure B3. Absorbance versus time data for 2.00 mM substrate concentration.

Table B5 shows the reaction rate data for the original sample.

Table B5. Reaction rate data for the original sample.

Substrate Concentration (mM)	Rate (V) (mM/sec) (x 10^4)
0.250	0.37
0.500	0.96
1.00	1.3
1.50	1.8
1.75	2.0
2.00	1.9

POLYMATH Results

Nonlinear regression (L-M)

Model: Rori = Vmax*S/(Km+S)

Variable	Ini guess	Value	95% confidence
Vmax	1	4.59E-04	6.03E-06
Km	1	2.1806002	0.0474467

Nonlinear regression settings
Max # iterations = 64

Precision
R^2 = 0.9671477
R^2adj = 0.9507216
Rmsd = 3.724E-06
Variance = 1.109E-10

General
Sample size = 4
Model vars = 2
Indep vars = 1
Iterations = 9

The k_{cat} value was determined using the equation 6 and was found to be 0.050 sec^{-1}. Similar calculations are done for the duplicate samples. The "Duplicate 1" sample values were eliminated due to low R^2 value and the 95% confidence was higher than the respective parameter values. The calculated values for the original and duplicate samples are shown in Table B6.

Table B6. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 7.80.

DL-BAPNA substrate	Original sample	Duplicate 1 sample	Duplicate 2 sample	Duplicate 3 sample	Standard Deviation
K_M (mM)	2.2	0.95	2.3	1.8	± 0.61
k_{cat} (sec^{-1})	0.050	0.039	0.067	0.056	± 0.011

4. 10 mM $MgCl_2$, July 21, 2004

A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure B4.

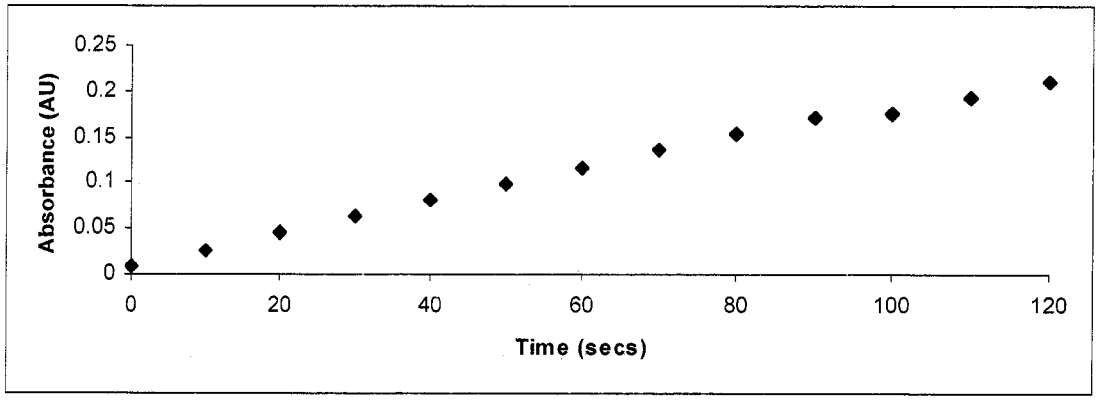

Figure B4. Absorbance versus time data for 2.00 mM substrate concentration.

Table B7 shows the reaction rate data for the original sample.

Table B7. Reaction rate data for the original sample.

Substrate Concentration (mM)	Rate (V) (mM/sec) (x 10^4)
0.250	0.58
0.500	1.2
1.00	1.4
1.50	1.9
1.75	1.8
2.00	1.9

POLYMATH Results

Nonlinear regression (L-M)

Model: Rori = Vmax*S/(Km+S)

Variable	Ini guess	Value	95% confidence
Vmax	1	2.727E-04	9.325E-05
Km	1	0.7763595	0.6934368

Nonlinear regression settings
Max # iterations = 64

Precision
R^2 = 0.9464837
R^2adj = 0.9331046
Rmsd = 4.665E-06
Variance = 1.959E-10

General
Sample size = 6
Model vars = 2
Indep vars = 1
Iterations = 9

The k_{cat} value was determined using the equation 6 and was found to be 0.029 sec^{-1}. Similar calculations are done for the duplicate samples. The calculated values for the original and duplicate samples are shown in Table B8.

Table B8. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 7.80.

DL-BAPNA substrate	Original sample	Duplicate 1 sample	Duplicate 2 sample	Duplicate 3 sample	Standard Deviation
K_M (mM)	0.78	2.2	0.87	1.6	± 0.67
k_{cat} (sec^{-1})	0.029	0.049	0.030	0.042	± 0.0097

5. 10 mM $CaCl_2$, July 22, 2004

A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure B5.

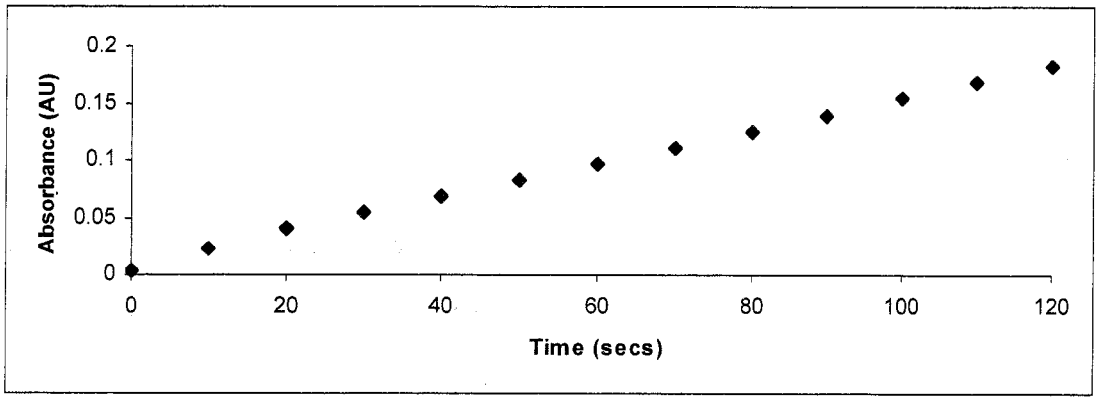

Figure B5. Absorbance versus time data for 2.00 mM substrate concentration.

Table B9 shows the reaction rate data for the original sample.

Table B9. Reaction rate data for the original sample.

Substrate Concentration (mM)	Rate (V) (mM/sec) (x 10^4)
0.250	0.41
0.500	0.81
0.750	0.88
1.00	1.2
1.50	1.5
1.75	1.5
2.00	1.7

POLYMATH Results
Nonlinear regression (L-M)

Model: Rori = Vmax*S/(Km+S)

Variable	Ini guess	Value	95% confidence
Vmax	1	2.803E-04	7.71E-05
Km	1	1.4191462	0.7545134

Nonlinear regression settings
Max # iterations = 64

Precision

R^2	= 0.9807682
R^2adj	= 0.9769219
Rmsd	= 2.196E-06
Variance	= 4.724E-11

General

Sample size	= 7
# Model vars	= 2
# Indep vars	= 1
# Iterations	= 7

The k_{cat} value was determined using the equation 6 and was found to be 0.031 sec^{-1}. Similar calculations are done for the duplicate samples. The calculated values for the original and duplicate samples are shown in Table B10.

Table B10. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 7.80.

DL-BAPNA substrate	Original sample	Duplicate 1 sample	Duplicate 2 sample	Duplicate 3 sample	Standard Deviation
K_M (mM)	1.4	1.4	1.6	0.98	± 0.26
k_{cat} (sec^{-1})	0.031	0.033	0.039	0.033	± 0.0035

6. 0.005 mM $CaCl_2$, July 22, 2004

A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure B6.

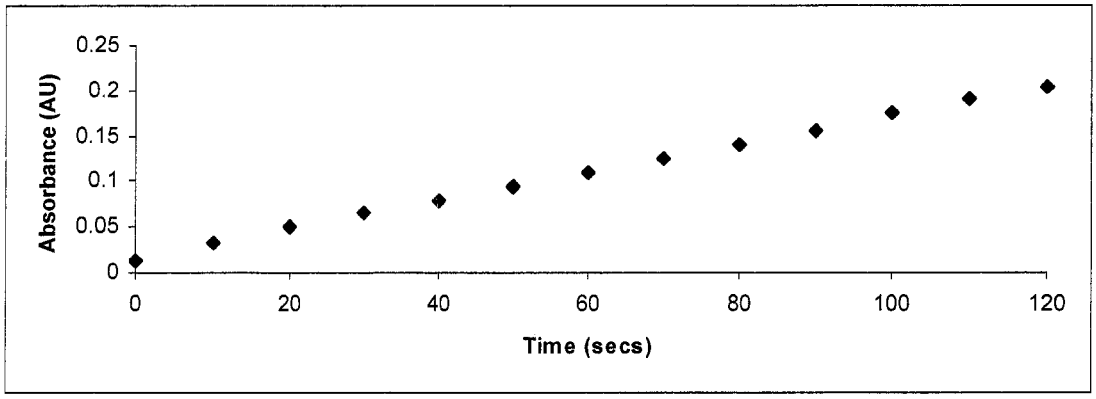

Figure B6. Absorbance versus time data for 2.00 mM substrate concentration.

Table B11 shows the reaction rate data for the original sample.

Table B11. Reaction rate data for the original sample.

Substrate Concentration (mM)	Rate (V) (mM/sec) (x 10^4)
0.250	0.41
0.500	0.81
0.750	0.88
1.00	1.2
1.50	1.5
1.75	1.5
2.00	1.7

POLYMATH Results

Nonlinear regression (L-M)

Model: Rori = Vmax*S/(Km+S)

Variable	Ini guess	Value	95% confidence
Vmax	1	2.39E-04	5.493E-06
Km	1	1.0834697	0.05526591

Nonlinear regression settings
Max # iterations = 64

Precision
R^2 = 0.9807682
R^2adj = 0.9769219
Rmsd = 2.196E-06
Variance = 4.724E-11

General
Sample size = 6
Model vars = 2
Indep vars = 1
Iterations = 6

The k_{cat} value was determined using the equation 6 and was found to be 0.026 sec^{-1}. Similar calculations are done for the duplicate samples. The calculated values for the original and duplicate samples are shown in Table B12.

Table B12. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 7.80.

DL-BAPNA substrate	Original sample	Duplicate 1 sample	Duplicate 2 sample	Duplicate 3 sample	Standard Deviation
K_M (mM)	1.1	2.1	0.49	0.35	± 0.79
k_{cat} (sec^{-1})	0.026	0.039	0.020	0.018	± 0.0095

7. 0.005 mM $CeCl_3$, July 22, 2004

A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure B7.

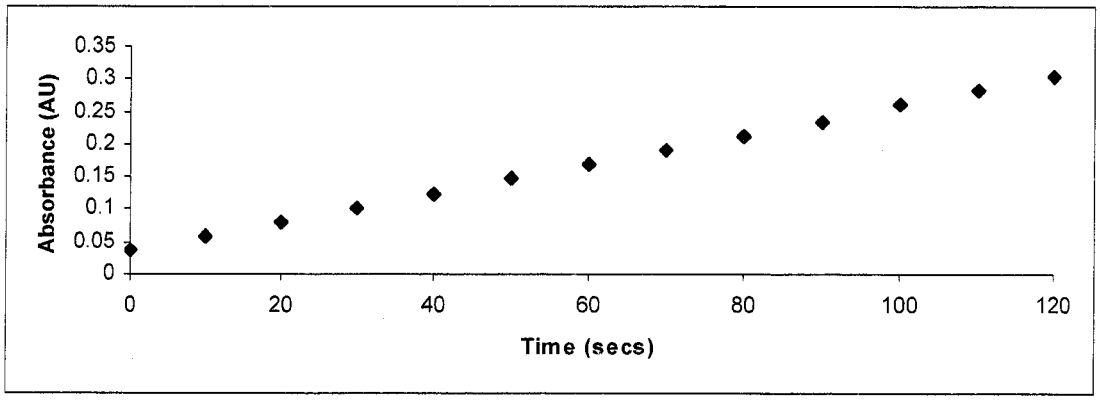

Figure B7. Absorbance versus time data for 2.00 mM substrate concentration.

Table B13 shows the reaction rate data for the original sample.

Table B13. Reaction rate data for the original sample.

Substrate Concentration (mM)	Rate (V) (mM/sec) (x 10^4)
0.500	1.3
0.750	1.9
1.00	1.7
1.50	2.2
1.75	2.7
2.00	2.7

POLYMATH Results
Nonlinear regression (L-M)

Model: Rori = Vmax*S/(Km+S)

Variable	Ini guess	Value	95% confidence
Vmax	1	3.789E-04	1.712E-04
Km	1	0.8948508	0.9801232

Nonlinear regression settings
Max # iterations = 64

Precision

R^2	= 0.8729086
R^2adj	= 0.8411357
Rmsd	= 6.895E-06
Variance	= 4.278E-10

General

Sample size	= 6
# Model vars	= 2
# Indep vars	= 1
# Iterations	= 9

The k_{cat} value was determined using the equation 6 and was found to be 0.042 sec^{-1}. Similar calculations are done for the duplicate samples. The calculated values for the original and duplicate samples are shown in Table B14.

Table B14. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 7.80.

DL-BAPNA substrate	Original sample	Duplicate 1 sample	Duplicate 2 sample	Duplicate 3 sample	Standard Deviation
K_M (mM)	0.89	1.3	0.94	0.97	± 0.19
k_{cat} (sec^{-1})	0.042	0.049	0.025	0.025	± 0.012

8. 0.5 mM $MgCl_2$, July 22, 2004

A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure B8.

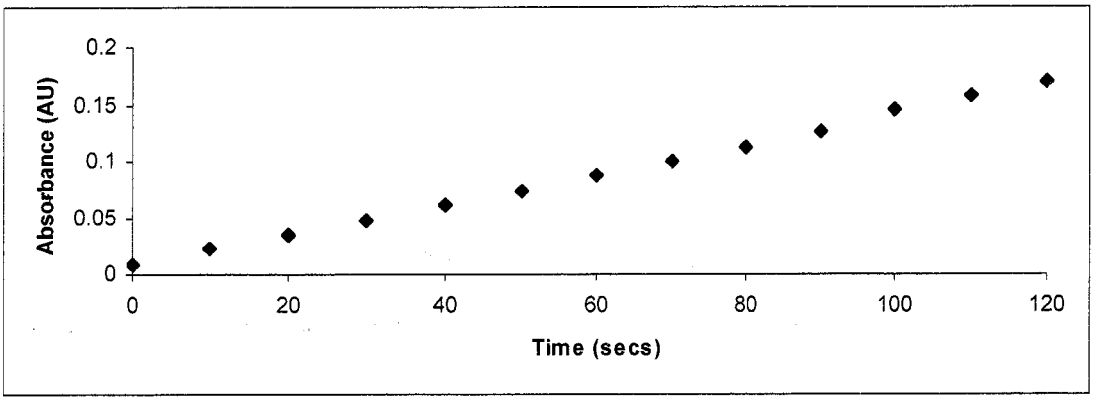

Figure B8. Absorbance versus time data for 2.00 mM substrate concentration.

Table B15 shows the reaction rate data for the original sample.

Table B15. Reaction rate data for the original sample.

Substrate Concentration (mM)	Rate (V) (mM/sec) (x 10^4)
0.500	0.24
0.750	1.2
1.00	1.4
1.50	1.5
1.75	1.9
2.00	2.4

POLYMATH Results
Nonlinear regression (L-M)

Model: Rori = Vmax*S/(Km+S)

Variable	Ini guess	Value	95% confidence
Vmax	1	2.551E-04	3.895E-07
Km	1	1.1982838	0.0039239

Nonlinear regression settings
Max # iterations = 64

Precision
R^2 = 0.9464837
R^2adj = 0.9331046
Rmsd = 4.665E-06
Variance = 1.959E-10

General
Sample size = 6
Model vars = 2
Indep vars = 1
Iterations = 9

The k_{cat} value was determined using the equation 6 and was found to be 0.029 sec^{-1}. Similar calculations are done for the duplicate samples. The calculated values for the original and duplicate samples are shown in Table B16.

Table B16. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 7.80.

DL-BAPNA substrate	Original sample	Duplicate 1 sample	Duplicate 2 sample	Duplicate 3 sample	Standard Deviation
K_M (mM)	1.2	0.91	1.1	0.67	± 0.24
k_{cat} (sec^{-1})	0.029	0.026	0.037	0.029	± 0.0047

9. 0.005 mM $MgCl_2$, July 23, 2004

A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure B9.

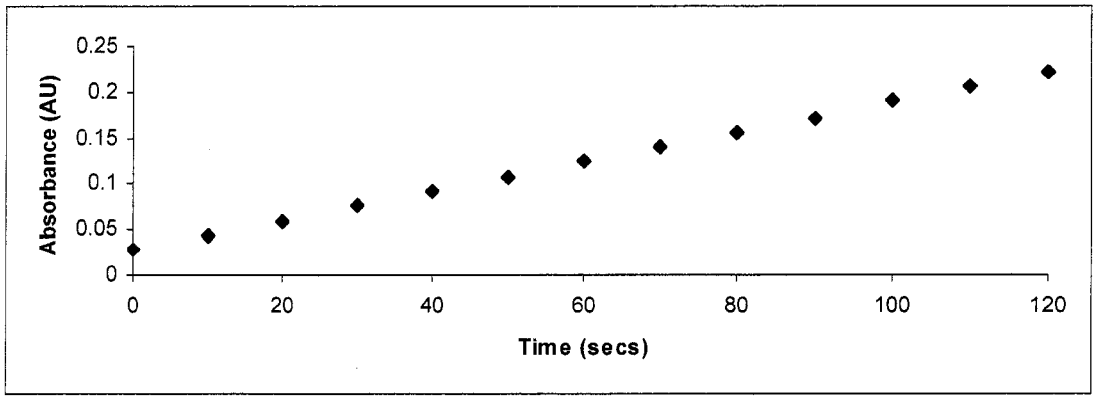

Figure B9. Absorbance versus time data for 2.00 mM substrate concentration.

Table B17 shows the reaction rate data for the original sample.

Table B17. Reaction rate data for the original sample.

Substrate Concentration (mM)	Rate (V) (mM/sec) (x 10^4)
0.500	0.96
0.750	1.1
1.00	1.3
1.50	1.4
1.75	1.7
2.00	1.8

POLYMATH Results
Nonlinear regression (L-M)

Model: Rori = Vmax*S/(Km+S)

Variable	Ini guess	Value	95% confidence
Vmax	1	2.352E-04	6.044E-05
Km	1	0.538735	0.4135588

Nonlinear regression settings
Max # iterations = 64

Precision

R^2	= 0.8930911
R^2adj	= 0.8717094
Rmsd	= 4.429E-06
Variance	= 1.922E-10

General

Sample size	= 7
# Model vars	= 2
# Indep vars	= 1
# Iterations	= 14

The k_{cat} value was determined using the equation 6 and was found to be 0.026 sec^{-1}. Similar calculations are done for the duplicate samples. The calculated values for the original and duplicate samples are shown in Table B18.

Table B18. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 7.80.

DL-BAPNA substrate	Original sample	Duplicate 1 sample	Duplicate 2 sample	Duplicate 3 sample	Standard Deviation
K_M (mM)	0.54	1.3	1.2	1.1	± 0.34
k_{cat} (sec^{-1})	0.026	0.036	0.039	0.036	± 0.0057

10. 10 mM $CeCl_3$, July 23, 2004

There was no activity for trypsin at 10.0 mM $CeCl_3$ concentration.

11. 50 mM $MnCl_2$, July 29, 2004

A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure B10.

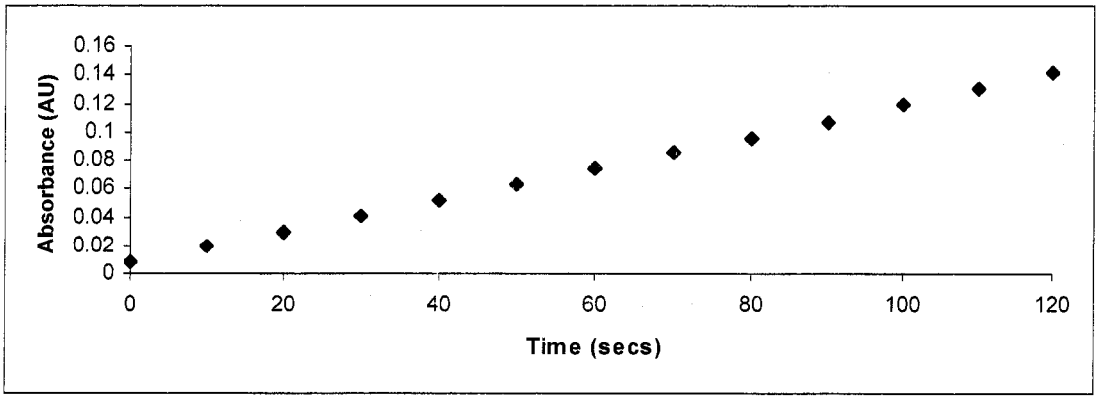

Figure B10. Absorbance versus time data for 2.00 mM substrate concentration.

Table B19 shows the reaction rate data for the original sample.

Table B19. Reaction rate data for the original sample.

Substrate Concentration (mM)	Rate (V) (mM/sec) (x 10^4)
0.250	0.50
0.500	0.74
0.750	0.79
1.00	0.91
1.50	1.0
1.75	1.0
2.00	1.3

POLYMATH Result
Nonlinear regression (L-M)

Model: Rori = Vmax*S/(Km+S)

Variable	Ini guess	Value	95% confidence
Vmax	1	1.494E-04	4.354E-05
Km	1	0.5761134	0.4852912

Nonlinear regression settings
Max # iterations = 64

Precision
R^2 = 0.8899597
R^2adj = 0.8679516
Rmsd = 3.028E-06
Variance = 8.985E-11

General
Sample size = 7
Model vars = 2
Indep vars = 1
Iterations = 10

The k_{cat} value was determined using the equation 6 and was found to be 0.016 sec^{-1}. Similar calculations are done for the duplicate sample. The calculated values for the original and duplicate sample are shown in Table B20.

Table B20. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 7.80.

DL-BAPNA substrate	Original sample	Duplicate sample	Standard Deviation
K_M (mM)	0.58	0.81	± 0.16
k_{cat} (sec^{-1})	0.016	0.019	± 0.0021

12. 50 mM KCl, July 29, 2004

A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure B11.

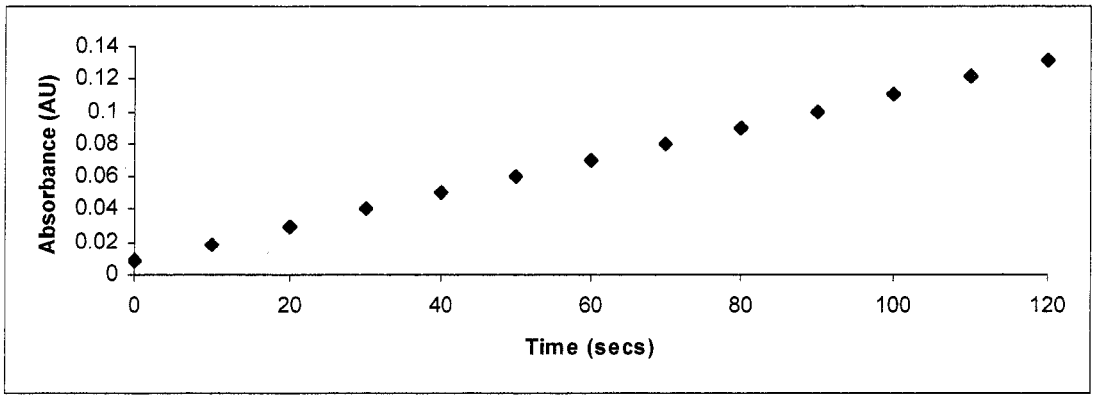

Figure B11. Absorbance versus time data for 2.00 mM substrate concentration.

Table B21 shows the reaction rate data for the original sample.

Table B21. Reaction rate data for the original sample.

Substrate Concentration (mM)	Rate (V) (mM/sec) (x 10^4)
0.250	0.66
0.500	0.74
0.750	0.75
1.00	1.0
1.50	1.3
1.75	1.4
2.00	1.2

POLYMATH Results

Nonlinear regression (L-M)

Model: Rori = Vmax*S/(Km+S)

Variable	Ini guess	Value	95% confidence
Vmax	1	1.642E-04	6.414E-05
Km	1	0.5682076	0.6458056

Nonlinear regression settings
Max # iterations = 64

Precision
R^2 = 0.8001795
R^2adj = 0.7602154
Rmsd = 4.51E-06
Variance = 1.993E-10

General
Sample size = 7
Model vars = 2
Indep vars = 1
Iterations = 11

The k_{cat} value was determined using the equation 6 and was found to be 0.018 sec^{-1}. Similar calculations are done for the duplicate sample. The calculated values for the original and duplicate sample are shown in Table B22.

Table B22. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 7.80.

DL-BAPNA substrate	Original sample	Duplicate sample	Standard deviation
K_M (mM)	0.57	0.76	± 0.13
k_{cat} (sec^{-1})	0.018	0.021	± 0.0021

13. 0.005 mM $CrCl_3$, July 29, 2004

A sample of the absorbance versus time data for 2.00 mM substrate concentration is shown in Figure B12.

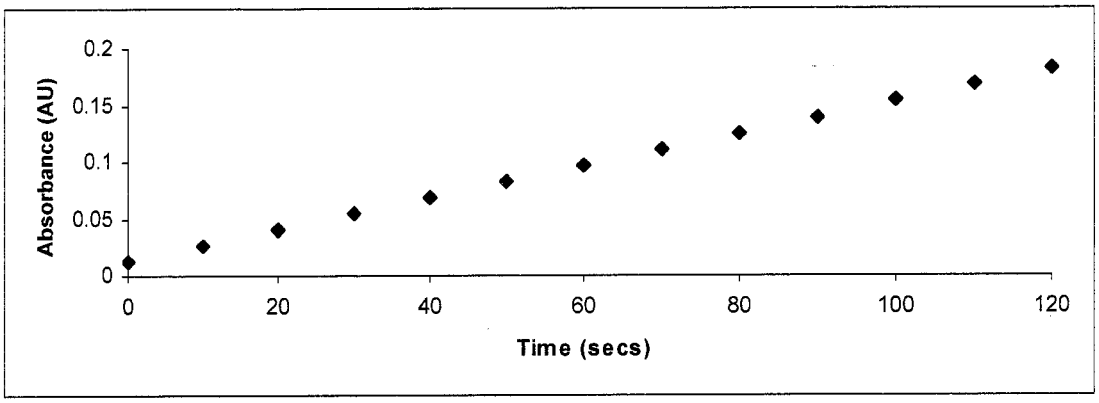

Figure B12. Absorbance versus time data for 2.00 mM substrate concentration.

Table B23 shows the reaction rate data for the original sample.

Table B23. Reaction rate data for the original sample.

Substrate Concentration (mM)	Rate (V) (mM/sec) (x 10^4)
0.250	0.56
0.500	1.1
0.750	1.2
1.00	1.3
1.50	1.7
1.75	1.8
2.00	1.7

POLYMATH Results

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Nonlinear regression (L-M)

Model: Rori = Vmax*S/(Km+S)

Variable	Ini guess	Value	95% confidence
Vmax	1	2.41E-04	5.895E-05
Km	1	0.7410514	0.4648124

Nonlinear regression settings

Max # iterations = 64

Precision

R^2	= 0.9521874
R^2adj	= 0.9426249
Rmsd	= 3.313E-06
Variance	= 1.076E-10

General

Sample size	= 7
# Model vars	= 2
# Indep vars	= 1
# Iterations	= 9

The k_{cat} value was determined using the equation 6 and was found to be 0.026 sec^{-1}. Similar calculations are done for the duplicate sample. The calculated values for the original and duplicate sample are shown in Table B24.

Table B24. Kinetic parameters for trypsin-catalyzed hydrolyses of DL- BAPNA at pH 7.80.

DL-BAPNA substrate	Original sample	Duplicate sample	Standard deviation
K_M (mM)	0.74	0.56	± 0.13
k_{cat} (sec^{-1})	0.026	0.025	± 0.0021